RESPONSE TO OFFICE ACTION

A. Status of the Claims

Claims 1-29 were pending at the time of the Action. Claim 29 is withdrawn following restriction.

Claims 1 and 15 are amended to clarify that the selection temperature is from about 28.5°C. to about 35 °C Support is found at paragraph [0019] of the specification.

New claims 30 and 31 are added. Support is found, at least, in Examples 3-5.

B. Objections to the Claims

Claims 1-28 are objected to as containing improper brackets and a non-conforming font. Applicants note that the listing of claims provided herein conforms with the accepted guidelines and lacks the brackets referred to in the Action. The corrections required no amendment to any claim language and thus no changes are marked or need to be marked in this regard. The objection is therefore believed moot and removal thereof is respectfully requested.

C. Rejection of Claims Under 35 U.S.C. §112, First Paragraph - Enablement

The Action rejects claims 1-17 as lacking enablement. In particular, the Action asserts that the specification does not reasonably provide enablement for all transformation methods, all cereal plants and all types of explants, nor for methods of *Agrobacterium* inoculation using 1 µl of *Agrobacterium* solution or filter paper saturated with *Agrobacterium*. Applicants respectfully traverse.

With respect to enablement of transformation methods, Applicants note both that the transformation method chosen is irrelevant to the enablement of the claims and further that

transformation methods are well known in the art. In particular, the claims relate to the surprising finding that selection of particular culture conditions improves the ability to obtain transformants from plant tissue into which a transgene has been inserted. However, the method by which the transgene is inserted is irrelevant to enablement, because the culturing takes place after transgene insertion. All that is relevant for purposes of enablement is that a tissue can be obtained for culturing comprising an introduced nucleic acid. This can be achieved by any technique.

It is a gross understatement to say that methods for insertion of a foreign gene into plant tissues are well known in the art. Applicants submit herewith as evidence of this a number of scientific publications relating to transformation in cereal plants. These publications provide just a few of the many examples of transformation and transgenic technologies available at the time the current application was filed. These publications include:

a. S. Tingay *et al.*, *Agrobacterium* tumefaciens-mediated Barley Transformation (*Plant J.* 11:1369-1376, 1997).

b. I. Roussy *et al.*, Transformation and Regeneration Capacities for Five Nordic Barley Elite Cultivars – Evaluation of Tissue Culture Response and Transient Expression (*Hereditas* 134:97-101, 2001).

c. Y. Hiei *et al.*, Efficient Transformation of Rice (*Oryza Sativ* L.) Mediated by *Agrobacterium* and Sequence Analysis of the Boundaries of the T-DNA (*Plant J.* 6:271-282, 1994).

d. M. Valdez *et al.*, Transgenic Central American, West African and Asian Elite Rice Varieties Resulting from Particle Bombardment of Foreign DNA into Mature Seed-derived Explants Utilizing Three Different Bombardment Devices, (*Annals of Botany* 82:795-801, 1998).

e. M. Cheng *et al.*, Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens* (*Plant Physiol.* 115:971-980, 1997).

f. A.M. Casas *et al.*, Transgenic Sorgham Plants via Microprojectile Bombardment (*Proc. Natl. Acad. Sci.*, USA, 90: 11212-11216, 1993).

g. K.A. Torbert *et al.*, Transformation of Oat Using Mature Embryo-Derived Tissue Cultures (*Crop Sci.* 38:226-231, 1998).

h. R.V. Sairam *et al.*, Shoot Meristem: An Ideal Explant for Zea mays L. Transformation (*Genome* 46:323-329, 2003).

Applicants note that, for example, the publications by S. Tingay *et a*l., Y. Hiei *et al.*, M. Cheng *et al.*, and R.V. Sairam *et al.* each provide detailed methods of introducing nucleic acids into cereals such as barley, rice, wheat and corn using *Agrobacterium* to mediate transformation. Also, the publications by I. Roussy *et al.*, M. Valdez *et al.*, A.M. Casas *et al. and* K.A. Torbert *et al.* describe methods for introducing nucleic acids into plant cells using bombardment or other non-biological methods. Further, the publication of R.V. Sairam *et al.* describes *Agrobacterium*-mediated transformation in maize shoot meristems, and points out at page 324, first column, first paragraph, that immature embryos, immature inflorescences, protoplasts, anthers, microspores, shoot apices, and suspension cultures have been used in previous studies.

As these publications show, methods of transforming maize and other cereals were well known at the time of filing the current application, and a variety of different plant tissues were known to be amenable to *Agrobacterium*-mediated transformation. There is, therefore, no basis to allege that the claims are not enabled for any given transformation method. Numerous transformation methods were well known in the art and the method chosen is irrelevant to the enablement of the claims. It was routine and well known in the art as of the filing date to insert a nucleic acid into maize or other cereals using *Agrobacterium* or non-biological means such as

particle bombardment. Thus, predictable methods of transforming cereals in addition to maize were already developed and in use.

Further, numerous plant tissues had been shown to be transformable, including immature embryos, immature inflorescences, protoplasts, anthers, microspores, shoot apices, and suspension cultures. As such, methods of transforming different tissues were known, and the use of such methods with maize and other cereals would merely involve standard transformation and culturing techniques well known in the art. Thus, the knowledge in the art coupled with the descriptions and examples in the current specification fully enable the claims.

With respect to the assertion in the Action that the working examples involving *Agrobacterium*-mediated transformation are not representative, Applicants note as indicated above that the selection of transformed cells takes place ***after*** a nucleic acid is inserted into plant cells. The manner in which the nucleic acid is inserted into the transformed cell is therefore ***not important*** for selection in accordance with the claims. This is shown, for example, in the publication of I. Roussy *et al.*, which compares *Agrobacterium* and particle bombardment methods in barley, and reports that cells transformed by either method were selected in the same way – transfer to CIM medium (page 98, second column). Also, the cited review by Hansen *et al.* (*Trends in Plant Science* 4:226-231) describes selection as an "important part of the transformation process," but does not point to any particular method of transformation as critical or special (page 229, column 2, under heading "Selection"). Because Applicants describe in detail how to transform cells using *Agrobacterium* or particle bombardment and these and other techniques for transformation were well known in the art, the claims are fully enabled for "inserting nucleic acid."

With respect to the assertion in the Action that regeneration of plants is not enabled, Applicants note that the submitted publications also establish that regeneration of plants from various explants was well known at the time the current application was filed. For example, the cited Tisserat reference (*Plant Cell Culture*, IRL Press, Oxford, pages 79-90) indicates that plants can be generated from multiple tissues, stating that "***[p]ractically any part*** of the plant can be ***successfully cultured in vitro and can regenerate plantlets*** provided the explant is obtained at the proper physiological stage of development" (page 81, line 3; emphasis added). While the reference indicates that the explant needs to be obtained at the proper physiological stage, the paper also demonstrates that such stages are known and that the identification of any given stage for a particular tissue is a routine matter. Thus, Applicants description and examples together with the knowledge in the art fully demonstrate the enablement for plant regeneration commensurate with the scope of the claims.

With regard to the methods of inoculating *Agrobacterium* using a 1 µl solution or a saturated filter paper, Applicants description of these inoculations fully enable the claims. As noted above, transformation with *Agrobacterium* is a well known technique routine in the art. The growth and preparation of various strains of *Agrobacterium* is standard practice, as is the preparation of various plant tissues for inoculation. Following inoculation, the efficiency of transformation can be analyzed by straight forward transient expression techniques, such as analysis of reporter gene expression, as described in the publications of Y. Hiei *et al.* and M. Cheng *et al.*, and in the cited B.R. Frame *et al.* reference *(Agrobacterium tumefaciens*-Mediated Transformation of Maize Embryos Using a Standard Binary Vector System, *Plant Physiology* 129:13-22, 2002; page 14, column 2, last paragraph). If changes in experimental conditions are desired, the impact of such changes on transformation efficiency can be readily assessed using

transient expression. Therefore, while some routine experimentation might be required, this would be routine in view of the detailed teaching in the specification and knowledge in the art.

As for the inclusion of surfactant in the inoculation medium, while a surfactant may enhance transformation efficiency under some conditions, successful transformation by no means require the presence of surfactant and no basis for so concluding is provided in the Action. For example, as described in the publication of R.V. Sairam *et al.*, maize shoot meristems were incubated with *Agrobacterium* in MS salts (Murashige and Skoog formulation), 1% w/v glucose and 200 μM acetosyringone to generate transformed explants (page 324, column 2, under the heading "Plant Transformation Method"). As shown in Table 3 of the cited Tisserat reference (page 84), MS salts do not contain a surfactant. Similarly, neither glucose nor acetosyringone is a surfactant. Thus, successful transformation of maize root meristems occurs without the inclusion of surfactant. Also, as described in the publication of M. Cheng *et al.*, different wheat tissues were inoculated with *Agrobacterium* in the presence or absence of a surfactant. While the efficiency of transformation was better in some cases with the surfactant, sufficient transformation occurred in the absence of the surfactant (page 973, column 2, last paragraph, to page 975, column 1, first paragraph, and Table 1). As these publications attest, a surfactant is not required for transformation. Thus, inoculating with 1 μl of *Agrobacterium* solution or with saturated filter paper are fully described for enablement purposes. Any fine tuning of the techniques would involve merely routine experimentation, as noted in the preceding paragraph.

By following established and well known procedures for transforming cereal tissues, either with *Agrobacterium* or through non-biological methods, and by following the teachings in the specification, a person of skill in the art can readily practice the methods set out in claims 1-

17. While some experimentation may be required, any experimentation would be routine given the substantial teaching in the specification and the knowledge in the art. In sum, Applicants have affirmatively demonstrated enablement of the claims and no basis to doubt the enablement has been provided. Removal of the rejection is thus respectfully requested.

D. Rejection Under 35 U.S.C. §102

The Action rejects claims 1, 6-10, 14-16, 20-24 and 28 as anticipated or obvious over Frame *et al.* in evidence of Zhao *et al.* (2001, Molecular Breeding 8:323-333). In particular, it is asserted that the references teach that selection of transformed cells is carried out at 28 °C.

Applicants note that the current claims 1 and 15 recite a temperature range from about 28.5°C to about 35 °C. The Action does not assert that the cited reference teaches or suggests temperatures above 28 °C. The cited references therefore does not teach all elements of the current claims and cannot form the basis for an anticipation rejection. The rejection is therefore believed moot and removal thereof is thus respectfully requested.

E. Rejection Under 35 U.S.C. §103

The Action rejects claims 1-10, 14-24 and 28 as obvious over Frame *et al.* in view of Zhao *et al.* In particular, it is asserted that different times of selection and different selection temperatures involve optimization of process parameters.

In response, Applicants note that the cited art is lacking any suggestion or motivation to select transformed cells in the temperature range defined by the claims, and thus there is no basis for an obviousness rejection. Specifically, nothing in the cited art suggests that benefit could be obtained by increasing selection temperatures or in particular by use of selection at 28.5°C or

higher. In contrast, Applicants have shown that such conditions may be used to achieve increased transformation frequency as seen in Example 8 and Table 3 of the specification. Indeed, other than mentioning a particular temperature used for infection or co-cultivation, the cited references do not mention selection temperature as a relevant parameter for transformation. Similarly, the review by Hansen *et al.* does not indicate that selection temperature should be studied or considered even though the review provides a detailed discussion about various steps and critical features of transformation systems. Further, in the publications of M. Cheng *et al.*, A.M Casas *et al.*, I. Roussy *et al.*, and R.V. Sairam *et al.*, which involve studies of factors affecting transformation in various cereals, selection temperature is not even considered. Thus, at the time of filing the current application, selection temperature was not regarded as an important parameter for investigation in studies of transformation efficiency.

With selection temperature considered unimportant, the prior art provides no reason to vary selection temperature or to try to optimize the selection temperature. As such, the prior art provides no motivation to select cells at higher temperatures. Moreover, the increase in transformation frequency upon selection at higher temperatures is unexpected considering that the prior art does not regard selection temperature as an important parameter for investigation. One of skill in the art could just have readily expected a decreased transformation efficiency.

In view of the foregoing, the claims are not properly rejected as obvious under §103 and removal of the rejection is thus respectfully requested.

CONCLUSION

In view of the foregoing, Applicants respectfully request favorable consideration of this case.

The Examiner is invited to contact the undersigned attorney at (512) 536-3085 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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TECHNICAL ADVANCE

Agrobacterium tumefaciens-mediated barley transformation

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Summary

Genetically transformed barley was produced by co-cultivating immature embryo explants with *Agrobacterium tumefaciens* carrying a binary vector coding for chimaeric bacterial genes, *bar* and *gus*, and selecting for bialaphos-resistant cultures from which plants were regenerated. Integration of both genes was confirmed by gel blot hybridization analysis of DNA from the transformed plants and their progenies. From 1282 embryos, plants were recovered for 54 independently transformed lines, giving a transformation efficiency of 4.2%. Transgene numbers in the different lines ranged from single copy insertion to at least ten copies. Sixteen out of 18 plants grown to maturity were fully fertile. Both marker genes, *bar* and *gus*, were expressed and co-segregated in the T_1 progeny plants. In the majority of cases, the genes showed Mendelian segregation predicted for transgene insertion at a single locus. In one family with multiple transgene insertions, molecular analysis of T_1 and T_2 plants suggested that the T-DNA had inserted at two unlinked loci.

Introduction

The development of an efficient method for genetic transformation is a prerequisite for its application for genetic studies and improvement of a given crop species. For barley, applications include genetic modification for improved agronomic performance and malting quality (McElroy and Jacobsen, 1995). Although transformation mediated by *Agrobacterium tumefaciens* has been applied to a wide range of broad-leaved (dicotyledonous) crop species, alternative approaches, such as biolistics and direct gene transfer to protoplasts (electroporation or polyethyleneglycol-mediated uptake), have been favoured for graminaceous monocots. This resulted in part from a prevailing belief that *Agrobacterium* delivery of DNA could not be used for the production of transgenic cereals (Potrykus, 1990), although a few studies provided evidence for *Agrobacterium*-mediated transformation of cereal tissues (Gould *et al.*, 1991; Mooney *et al.*, 1991; Raineri *et al.*, 1990). Substantive confirmation is now accumulating that stably transformed cereal plants can be produced using *Agrobacterium* as a vector (Chan *et al.*, 1992, 1993; Hiei *et al.*, 1994; Ishida *et al.*, 1996; Rashid *et al.*, 1996).

For barley, the most successful method for transformation to date has been microprojectile bombardment into immature zygotic embryos, callus derived from immature embryos and microspore-derived embryos (Wan and Lemaux, 1994). Microprojectile bombardment of barley microspores has also resulted in the regeneration of fertile transgenic plants (Jähne *et al.*, 1994); however, the efficiency of this method of transformation appears to be low. Alternatively, transformation can be achieved by direct gene transfer to barley protoplasts (Funatsuki *et al.*, 1995), although this requires the time-consuming initiation of embryogenic suspension cultures. Compared to these direct gene transfer methods, *Agrobacterium*-mediated transformation offers a number of advantages, including defined transgene integration, potentially low copy number and preferential integration into transcriptionally active regions of the chromosome (Czernilofsky *et al.*, 1986; Koncz *et al.*, 1989). DNA transfer by means of *Agrobacterium* has been attempted in the past for barley, and whereas agroinfection and T-DNA transfer have been demonstrated, integration of the DNA into the genome was not shown (Boulton *et al.*, 1989; Creissen *et al.*, 1990; Deng *et al.*, 1990).

In this study, we demonstrate stable barley transformation by co-cultivation of immature embryos with *Agrobacterium tumefaciens*. DNA hybridization analyses confirm the presence of the transferred *bar* and *gus* genes. Expression of both genes is confirmed in the T_0 and T_1 generations.

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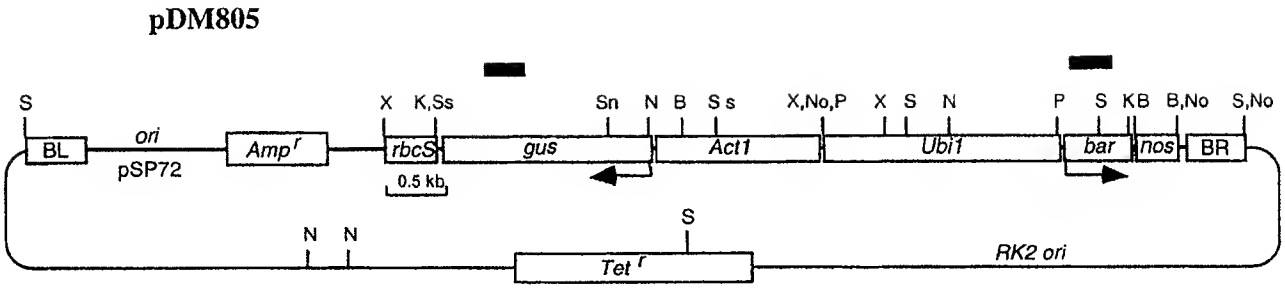

Figure 1. Structure and restriction map of the cereal transformation vector pDM805.
The locations of the probes used for *gus* and *bar* are indicated in each case by a line above the gene. Abbreviations: *Act1*, promoter, first exon and intron of the rice actin 1 gene; *gus*, coding region of the *E.coli* β-glucuronidase gene; *rbcS*, 3' transcript termination region of the rice rubisco gene; *Ubi1*, promoter, first exon and first intron of the maize ubiquitin 1 gene; *bar*, coding region of *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene; nos, 3' transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene; *Amp*^{r}, ampicillin resistence gene of pSP72; BL, T-DNA left border sequence; BR, T-DNA right border sequence; *Tet*^{r}, tetracycline resistance gene. The pSP72 and pTAB5 regions are not drawn to scale. Restriction sites are abbreviated as follows: B, *Bam*HI; K, *Kpn*I; N, *Nco*I; No, *Not*I; P, *Pst*I; S, *Sal*I; Sn, *Sna*BI; Ss, *Sac*I; X, *Xho*I.

Results

Production of plants, expressing bar *and* gus*, from immature embryos co-cultivated with* Agrobacterium tumefaciens

Preliminary tests were conducted to compare the responses of embryos from which the embryonic axis was removed with the response of embryos left intact. At the same time, we assessed the effect of injuring the embryo explants by shooting with gold microprojectiles prior to inoculation with *Agrobacterium*. In all experiments, *Agrobacterium tumefaciens* AGL1 (pDM805) was used, where pDM805 is a binary vector containing the the *Act1.gus.rbcS* reporter gene and the *Ubi1.bar.nos* selectable marker gene (Figure 1). Following co-cultivation with A. *tumefaciens*, the explants were cultured on callus induction medium containing bialaphos (3 mg l^{-1}) to inhibit growth of non-transformed barley cells, and supplemented with Timentin TM (150 mg l^{-1}) to inhibit bacterial growth. Under these conditions, callus developed over the whole surface of the embryo in all treatments, with the most vigorous growth of embryogenic tissue occurring around the periphery of the scutellum. After the first round of selection, 10–12 days, embryos had expanded in size, and development of smooth embryogenic callus was evident. The expanded embryos were then each cut into eight to 12 small pieces and transferred to fresh selection medium on which a proportion of the tissue maintained vigorous growth (Figure 2). During subsequent rounds of selection, proliferating tissue was cut up further, and browning or non-embryogenic tissues were discarded. Bialaphos-resistant callus was obtained at low frequencies from intact embryos that were not injured by a biolistic treatment. However, in all the following experiments, the embryo axes were removed, and the scutellar tissue was shot with gold microprojectiles, since after 6 weeks on selection, the highest proportions of bialaphos-resistant callus, from up to 35% of treated embryos, were obtained with this treatment.

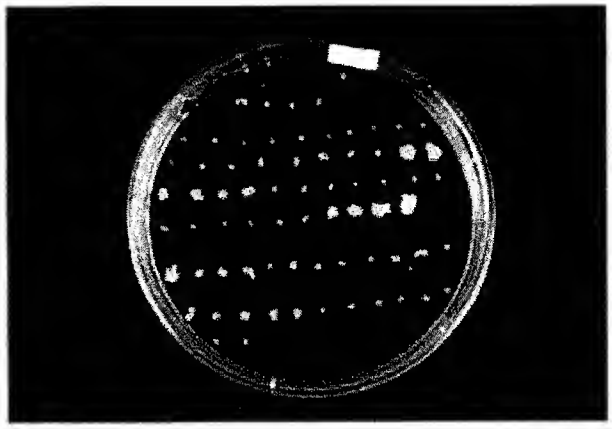

Figure 2. Barley callus at the second round of selection following co-cultivation with *Agrobacterium tumefaciens*.
Each line of callus pieces derives from a single embryo. Tissues showing vigorous growth can be distinguished from those for which growth is inhibited by bialaphos in the culture medium.

After 6–8 weeks on selection with bialaphos, embryogenic tissue was transferred to regeneration medium supplemented with 3 mg l^{-1} bialaphos. In the first experiment (Experiment I), bialaphos-resistant callus lines were selected from 454 embryos treated with A. *tumefaciens*. A total of 18 plantlets were regenerated from these callus lines, which derived from only two embryos, and all but two of these T_0 plants tested positive for both PAT and GUS activities (Table 1). The molecular analysis (detailed in the next section) of these plants showed that they correspond to at least nine independent transformation events, giving a frequency of 1.7% (individual transformation events per 100 embryos co-cultivated with A. *tumefaciens*). In a subsequent experiment (Experiment II), bialaphos-resistant callus lines and plants were recovered at a higher frequency of 3.6% (14 independent lines from 387 embryos, each derived from a different embryo)

Table 1. Transformants obtained in Experiment I. Expression of marker transgenes, estimated number of insertions of *gus* gene from DNA hybridization analysis of T_0 plants, and inheritance of bialaphos resistance in the T_1 generation

Primary transformants (T_0)				Bialaphos resistance in T_1 embryos		
	PAT activity	GUS activity	Minimum number of genes (*gus*) in T_0	Resistant	Sensitive	Segregation ratio[a] (χ^2 value)
GP[b] (control)	–	–	0	0	43	
I-1	++	++	3	111	46	3:1 (1.54, $P > 0.2$)
I-2	+	++	1	122	45	3:1 (0.34, $P > 0.5$)
I-3	++	++	5	35	33	
I-4	–	++	10	0	54	
I-5	+	++	1	80	35	3:1 (1.81, $P > 0.1$)
I-6	++	++	5	83	12	
I-7	+	+	1	72	6	15:1 (0.28, $P > 0.5$)
I-8	+	+	2	55	19	3:1 (0.02, $P > 0.8$)
I-9	++	++	4	23	7	3:1 (0.04, $P > 0.8$)
I-10	+	+	1	73	27	3:1 (0.21, $P > 0.5$)
I-11	++	++	5	64	3	15:1 (0.36, $P > 0.5$)
I-12	+	+	1	Not tested	Not tested	
I-13	+	+	1	102	27	3:1 (1.14, $P > 0.2$)
I-14	++	+	3	Not tested	Not tested	
I-15	–	++	10	0	89	
I-16	++	++	1	146	35	3:1 (3.10, $P > 0.1$)
I-17	+	+	1	64	18	3:1 (0.41, $P > 0.5$)
I-18	++	++	4	136	21	

For determination of PAT in leaf samples, ++ denotes strong activity with an intense band corresponding to acetylated product of the enzyme, + weak activity, and – denotes no detectable product.
For the GUS assay, ++ denotes strong blue colouration within 6 h of adding X-Gluc substrate, + denotes weak activity, and – denotes no detectable activty in leaf tissue samples.
[a]Segregation ratios are given for one or two dominant (active) loci, together with the corresponding χ^2 value.
[b]Golden promise, untransformed parent.

when excess *Agrobacterium* suspension was drained from embryos before plating. This prevented excessive overgrowth of bacteria on the embryos, which causes them to brown and results in reduced or no callus initiation. Using this procedure, transformed barley plants have been produced in three subsequent experiments at a mean frequency of 7.0% (31 independent lines from 441 embryos).

Molecular analysis of T_0 plants

The binary plasmid vector pDM805 was restricted with *Bam*HI, which generates one internal 3.7-kb fragment containing the *bar* gene. Hybridization with the *bar* gene probe detected one 3.7-kb fragment in *Bam*HI-restricted genomic DNA from all 18 transformants from Experiment I, suggesting that the *bar* gene was successfully transferred to all tested plants. A variation in the signal intensity between the lanes (Figure 3b) was observed, indicative of a varying number of copies of the T-DNA being present in the individual transformants.To address this possibility, the filter was stripped and probed again with a *gus* gene probe.The banding pattern observed indicated that different plants contained between 1 and up to at least 10 copies

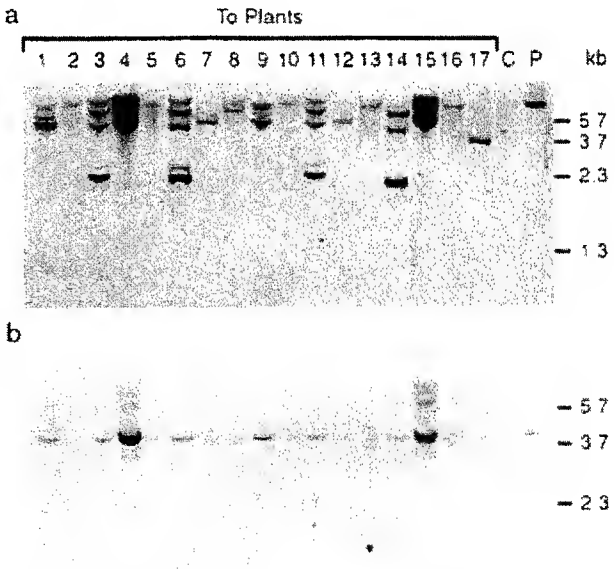

Figure 3. DNA gel blot hybridization analysis of transformed barley plants (T_0).
DNA from non-transgenic barley (C), purified plasmid pDM805 (P), and trensforments (tracks 1–17) was digested with *Bam*HI, frectioneted by electrophoresis, transferred to nylon membrane and hybridized with the *gus* probe (a) or the *ber* probe (b). The numbers of the plants correspond to those in Teble 1 for Experiment I (I-1 to I-17).

Table 2. Transformants obtained in Experiment II. Expression of marker transgenes and estimate of number of insertions of *gus* gene from hybridization analysis of T_0 plants

Primary transformants (T_0)	PAT activity	GUS activity	Minimum number of genes (gus) in T_0
GP (control)	–	–	0
II-1	++	–	4
II-2	+	++	1
II-3	+	++	1
II-4	–	+	1
II-5	+	++	2
II-6	++	+	6
II-7	+	–	1
II-8	++	–	1[a]
II-9	++	+	2

For determination of PAT in leaf samples, ++ denotes strong activity with an intense band corresponding to acetylated product of the enzyme, + weak activity, and – denotes no detectable product.
For the GUS assay, ++ denotes strong blue coloration within 6 h of adding X-Gluc substrate, + denotes weak activity, and – denotes no detectable activty in leaf tissue samples.
[a]In this line, a single band of less than 1 kb was detected for *gus*.

of the insert (Figure 3a and Table 1). It was also possible to deduce from this hybridization analysis that the plants were either independent transformants or fell into families with several clones probably arising from the same transformation event. The plants are grouped as follows: plant I-1 with at least three copies of the *gus* gene; I-2, I-5, I-10, I-13, I-16 with a single copy; I-3, I-6, I-11 with at least five copies; I-4, I-15 with at least 10 copies; I-7 with at least one copy; I-8 with two copies; I-9, I-18 (not shown) with at least four copies; I-12 with a single copy; and I-17 with a single copy. A single embryo explant gave rise to all these transformed plants, except for I-17. A total of nine independent transgenic events were thus detected from Experiment I. For Experiment II, the same molecular analysis revealed 14 independent transformation events, based on distinct banding patterns for the *gus* gene. Data for a set of plants from this experiment are shown in Table 2.

In the majority of independent transformants, the *gus* probe revealed bands greater than 5 kb in size, indicating that the whole of the T-DNA had integrated. An exception is plant I-17, which produced a smaller *gus* band (Figure 3a), but showed activity for both GUS and PAT (Table 1).

Inheritance in T_1 generation

Sixteen out of the 18 plants recovered in Experiment I were fully fertile. From these self-pollinated T_0 plants, representing seven independent transformation events, immature embryos were isolated and plated on to FHG

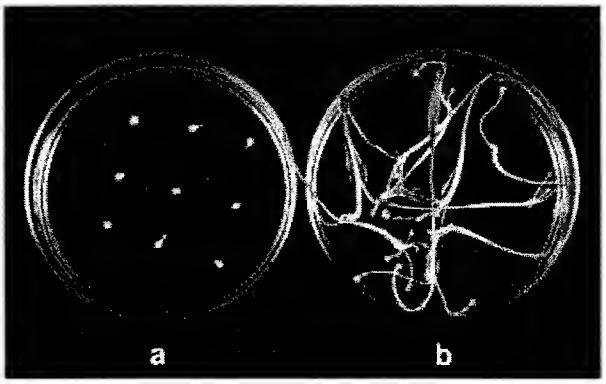

Figure 4. Barley embryos after 11 deys on medium containing 3 mg l^{-1} bialaphos.
Embryos from Golden Promise parent have failed to grow (a), whereas those from T_2 line I-6-4 have germinated uniformly (b).

medium supplemented with 3 mg l^{-1} bialaphos to examine inheritance of the herbicide resistance. After 4–5 days under fluorescent lighting, embryos were scored as being resistant if a green shoot and root system had emerged, whereas control embryos failed to germinate under these conditions (Figure 4). The results, given in Table 1, show that, for T_0 plants I-1, I-2, I-5, I-8, I-9, I-10, I-13, and I-17, the T_1 progenies conform to a 3:1 segregation for resistance:-sensitivity to bialaphos. For other T_0 plants, the numbers of sensitive plants are fewer than would be expected for a single locus for bialaphos resistance, raising the possibility of insertion of an active *bar* gene at more than one locus. The progeny of I-3 showed an approximately 1:1 segregation for bialaphos resistance, which does not conform to a Mendelian expectation. For plants I-4 and I-15 with apparently high copy insertion of the *bar* gene and no detectable PAT activity in leaf tissue, no resistance to bialaphos was detected among a total of 143 T_1 embryos tested.

Molecular analysis of T_1 plants

A selection of T_1 plants was chosen for further analysis using probes for *bar* and *gus*. These were derived from T_0 plants I-1, I-3, I-6, and I-16, and were also tested for PAT and GUS activities. A sample of the results in Figure 5 shows evidence for stable integration and inheritance of the transgenes at discrete loci. The plants represented by tracks 1 and 2 are selfed progenies of plant I-1. In one case, no hybridization is detected, but in the second, the banding pattern for *bar* and *gus* is identical to that seen for the T_0 plant, and this plant scored positive for both PAT and GUS activity in leaf tissue. Track 3 is a single derivative of plant I-3 with no apparent hybridization, and the plant was also negative for both PAT and GUS in leaf tissue. The following lanes (tracks 4–12) are of DNA isolated from nine individual T_1 plants derived from plant I-6. In this family,

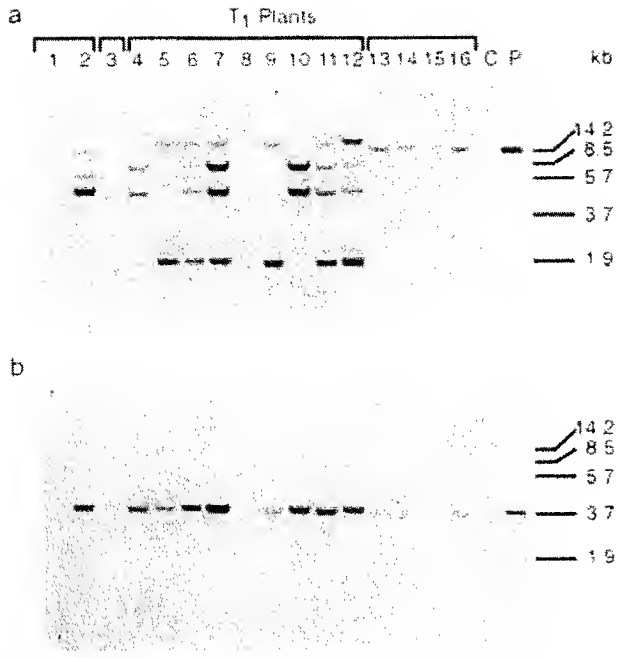

Figure 5. DNA gel blot hybridization analysis of T_1 progeny plants.
DNA from non-transgenic barley (C), purified plasmid pDM805 (P) and T_1 barley plants (1–16) was digested with *Bam*HI, fractionated by electrophoresis, transferred to nylon membrane and hybridized with the *gus* probe (a) or the *bar* probe (b). The plants used are self-pollinated progenies of T_0 plants I-1 (tracks 1 and 2), I-3 (track 3), I-6 (tracks 4–12) and I-16 (tracks 13–16).

the bands for *gus* in the T_1 plants correspond to those in the T_0 plant, but the uppermost and lowest bands segregate independently from the two middle bands (Figure 5a), indicating that the T-DNA has been inserted at more than one locus. Eleven out of 13 plants examined from this family were scored as positive for PAT and GUS; the two exceptions (one is represented by track 8) had no bands for *bar* and *gus*, as well as no detectable activity of these enzymes in leaf tissue. A simpler inheritance pattern is seen for the T_1 plants derived from plant I-16 (tracks 13–16), where the insertion appears to have occurred at a single locus. In all cases examined, the sizes of the bands revealed with the probe for the *gus* gene for the T_1 plants correspond to those observed for the T_0 plant; no new bands were detected.

The presence of *bar* and *gus* was correlated with the activities of PAT and GUS in leaf tissue for all four T_1 families examined. The two genes were found to be tightly linked, both for presence and expression. Eight out of 53 T_1 plants were found to lack both genes according to the DNA hybridization analysis, and all scored as negative for PAT and GUS. Of the remaining 45 plants with bands corresponding to *bar* and *gus*, all but three were positive for both enzyme activities. One, from I-16, was scored as positive for GUS but negative for PAT, even though the 3.7-kb hybridizing fragment was present, whereas two plants from I-1 were negative for GUS but positive for PAT.

Molecular analysis of T_2 plants

An analysis was conducted of progenies of one set of self-pollinated T_1 plants from line I-6 in which there were four bands for *gus*, where the uppermost and lowest bands segregated independently from the two middle bands. Hybridization analysis of these T_2 plants showed that the two sets of bands continued to segregate independently (data not shown), suggesting that the T-DNA is inserted at two unlinked loci. The results are also consistent with the relative intensities of hybridization seen for the T_1 plants, illustrated for plants I-6-4 and I-6-9 (Figure 5, tracks 7 and 12, respectively). Thus, plant I-6-9 shows weaker hybridization for the middle bands and appears to be hemizygous for the corresponding locus, as in the T_2 progeny the middle bands were absent in two of six plants examined, whereas all had the uppermost and lowest bands. Plant I-6-4, however, shows stronger hybridization of the middle bands and is probably homozygous for both loci since eight T_2 progeny plants had all four bands.

Discussion

This work represents a first report for barley that *Agrobacterium tumefaciens* can provide an effective method for transformation, and it follows from the convincing demonstrations of *Agrobacterium*-mediated transformation of rice and maize (Hiei *et al.*, 1994; Ishida *et al.*, 1996). Note that no special treatments, such as application of acetosyringone to induce the *vir* genes of *Agrobacterium* were applied, although it was found that wounding the cultured barley embryos with a shot of gold particles and removal of the axis enhanced the recovery of transformed tissue. This follows earlier studies where transformation was enhanced by bombarding tissues prior to application of *Agrobacterium* (Bidney *et al.*, 1992; Knittel *et al.*, 1994). There is no evidence that the mechanism of DNA transfer for cereals is any different to that observed for dicotyledonous plant species within the natural host range of *Agrobacterium tumefaciens*. The molecular analysis provides data that are consistent with T-DNA transfer from right border to left border (Zambryski, 1988), although bialaphos selection may have favoured segments close to the right border, where the selectable marker gene *bar* is located.

We have shown that two marker genes, *bar* and *gus*, on plasmid pDM805 are reproducibly transferred from *Agrobacterium* and stably integrated into the barley genome. The two genes continued to be tightly linked, and the corresponding enzymes PAT and GUS were detected in the majority of the transformed plants. The DNA hybridization analysis revealed variation in the number of copies of genes integrated, from one to more than 10 copies. Whereas no PAT activity was detected in one family with a very high copy number of *bar* and *gus*, there is no

clear correlation between copy number and the levels of transgene expression observed. Inheritance studies showed that both genes are transmitted to progeny, and behave as insertions at one or two loci.

The apparent difficulties encountered in transforming cereals with *Agrobacterium* led Potrykus (1990) to conclude that this approach had very little potential for cereals. We would ascribe the recent success with cereals to a combination of factors, including the choice of a supervirulent *Agrobacterium* strain and the availability of an appropriate starting material which is capable of active cell division leading to efficient plant regeneration. When compared to the 35S promoter from cauliflower mosaic virus, the use of the promoter from the maize ubiquitin gene, *Ubi1* (Christensen *et al.*, 1992), to drive the selectable marker gene has given higher frequencies of recovery of transformed callus in rice following microprojectile bombardment (Li *et al.*, 1997). The presence of the *Ubi1* promoter in pDM805 therefore may have also assisted in the recovery of transformed barley cultures in our experiments. We suggest that this plasmid would be suitable for transformation of a range of cereals and grasses.

The frequencies of transformation obtained in this study are comparable to the best that can be achieved currently with microprojectile bombardment (Wan and Lemaux, 1994). Significantly, we have been able to produce multiple independent transformants from a single embryo explant, indicating that further increases in transformation efficiencies can be anticipated with a refinement of the method.

Experimental procedures

Plant material

Plants of the spring barley cultivar Golden Promise were grown in growth cabinets, at 18°C during a 16-h light period and at 13°C for an 8-h dark period. Plants were fertilized with Osmocote (Scotts, Castle Hill, Australia) at the time of planting and then weekly with Aquasol (Hortico Pty. Ltd, Laverton North, Australia).

Immature embryos

Spikes of barley were harvested when the immature embryos were between 1.5 mm and 2.5 mm in length. The developing caryopses were sterilized for 10 min in a solution of sodium hypochlorite (Marvo-Linn Bleach) containing 1% w/v chlorine, then rinsed four times in sterile, distilled water. Immature embryos were excised from the young caryopses, and the embryonic axis was removed with a sharp scalpel blade. The explants were placed scutellum-side up on callus induction medium (Wan and Lemaux, 1994). Embryos were incubated at 24°C in the dark during co-cultivation and subsequent periods of culture.

Agrobacterium *strain and plasmid vector*

Agrobacterium tumefaciens strain AGL1 (Lazo *et al.*, 1991) was used in transformation experiments. pDM805 (Figure 1) was constructed by replacing the chimeric kanamycin resistance gene in the binary vector pTAB5 (Tabe *et al.*, 1995) with the complete sequence of the transformation vector pDM803 (D. McElroy, unpublished). Plasmid pDM803 contains: an *E. coli* origin of replication; an ampicillin resistance gene; a chimeric *Streptomyces hygroscopicus* phosphinothricin acetyl transferase (*bar*) gene (Thompson *et al.*, 1987), under the control of the promoter and first intron from the maize ubiquitin 1 (*Ubi1*) gene (Christensen *et al.*, 1992) and a transcription terminator sequence from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene (Bevan *et al.*, 1983); and a chimeric *E. coli* β-glucuronidase gene *uidA*, here referred to as *gus* (Jefferson *et al.*, 1987), under the control of the promoter and first intron from the rice actin 1 (*Act1*) gene (McElroy *et al.*, 1991) and a transcription terminator sequence from the rice small subunit of ribulose bis-phosphate carboxylase/oxygenase (*rbcS*) gene (Xie *et al.*, 1987). The 8.95-kb pDM803 vector was linearized by *Cla*I digestion and made blunt-ended by treatment with Klenow enzyme. The 9.2-kb binary vector from pTAB5 was isolated by digestion with *Bam*H1 and *Eco*R1 and made blunt-ended by Klenow treatment. The linearized pDM803 sequence was then cloned into the binary vector sequence from pTAB5 to create the 18.15-kb vector pDM805. The plasmid pDM805 was mobilized into AGL1 from *E. coli* by triparental mating.

Transformation

Agrobacterium tumefaciens AGL1 (pDM805) was grown from a single colony in MG/L medium (Garfinkel and Nester, 1980) supplemented with 20 mg l^{-1} rifampicin and 5 mg l^{-1} tetracycline, for 40 h at 27°C. *Agrobacterium tumefaciens* AGL1 (pDM805) was grown from a single colony in MG/L medium (Garfinkel and Nester, 1980) supplemented with 20 mg/L rifampicin and 5 mg/L tetracycline, for 40 h at 27°C. MG/L was prepared as a liquid broth with mannitol 5 g l^{-1}, L-glutamic acid 1 g l^{-1}, KH_2PO_4 250 mg l^{-1}, NaCl 100 mg l^{-1}, $MgSO_4$, $7H_20$ 100 mg l^{-1}, biotin 1 μg l^{-1}, tryptone 5 g l^{-1}, yeast extract 2.5 g l^{-1}, with pH adjusted to 7.0. A standard inoculum was prepared by adding 200 μl of culture to 200 μl of 15% aqueous glycerol in an Eppendorf tube, and kept at room temperature for 6 h before being transferred to −80°C. A full strength inoculum, approximately 2.8×10^9 bacterial cells ml^{-1}, was obtained by growing the standard inoculum in 10 ml of MG/L for 24 h at 27°C.

Immature embryos, the day after isolation, were injured by shooting the scutellum surface. Fifty to 70 embryos were used per shot, with 0.1 mg or 0.6 mg of gold particles (1.0 μm, Bio-Rad, Regents Park, Australia), using a Bio-Rad PDS-1000 Biolistic device with an 1100 p.s.i. rupture disc.

Following shooting, the embryos were immersed in the full strength *Agrobacterium* suspension and then immediately transferred, without rinsing, with the scutellar surface placed in contact with the callus induction medium. Plates were incubated at 24°C in darkness for 2 or 3 days. After the co-cultivation, embryos were transferred directly to callus induction medium supplemented with 3 mg l^{-1} bialaphos and 150 mg l^{-1} TimentinTM (SmithKline Beecham, Australia). The selection process, as described by Wan and Lemaux (1994), occurred for up to 8 weeks. Resistant embryogenic callus lines were transferred to FHG medium (Hunter, 1988) supplemented with 1 mg l^{-1} 6-BA, 3 mg l^{-1} bialaphos, and solidified with 3 g l^{-1} phytagel (Sigma), and incubated at 24°C under fluorescent lights (16 h day^{-1}). Regenerating plantlets were transferred to hormone-free callus induction medium with 1 mg l^{-1} bialaphos. After development of a root system, plantlets were transferred to soil and placed in growth cabinets set at 18°C 8 h

day/13°C night for 4 weeks, then transferred to 16-h day cabinets. Regenerants grew to maturity and self-pollinated.

Enzyme assays

PAT activity was determined using a modified thin-layer chromatographic assay (Schroeder *et al.*, 1993). For barley leaf extracts, 2.5 µl (0.05 µCi) of [1-^{14}C]acetyl-CoA (50–60 mCi mmol^{-1}, Amersham) was used per reaction. GUS activity was assayed histochemically in leaf tissue using X-Gluc substrate (Jefferson *et al.*, 1987).

Isolation of DNA and Southern hybridization

Genomic DNA was isolated from leaf tissue of individual regenerated plants essentially as described in Martienssen *et al.* (1989). Ten to 15 µg of genomic DNA was restricted with *Bam*HI restriction enzyme (NEB BioLabs). The restricted DNA was size-fractionated by agarose gel electrophoresis and transferred to positively charged nylon membrane, according to the method outlined in the DIG System User's Guide (Boehringer-Mannheim). The conditions for filter hybridization and post-hybridization washes were essentially as described by Engler-Blum *et al.* (1993), with two modifications: the concentration of SDS in the hybridization buffer was 15%, and post-detection washes were done in 0.02 M Tris–HCl instead of 0.1 M maleic acid. The probes were generated by PCR amplification using two sets of primers, PRBar5' (GGATCTACCATGAGCCCAGA) and PRBar3' (TGCCTCCAGGGACTTCAG) for the *bar* gene and GGUS5'(TAGAAACCCCAACCCGTGAAA) and GGUS3' (TGGCGTATAGCCGCCCTGATG) for the *gus* gene. DIG dUTP was incorporated into the amplified PCR products of 357 bp (*bar*) and 326 bp (*gus*). Filters were pre-hybridized for 3 h at 66.4°C (*gus*) or 72°C (*bar*), denatured probe was added and hybridization continued overnight. Filters were washed at 64°C (*gus*) or 70°C (*bar*) for 3 × 20 min with 20 mM Na_2HPO_4, 1m M EDTA, 1% SDS. Before detection, filters were washed as outlined above and blocked with 0.5% blocking reagent at room temperature. The detection was as described in the DIG users manual using the recommended dilution of anti-DIG-AP conjugate (Boehringer-Mannheim). CDP Star was used as chemiluminescent substrate and signals were detected by autoradiography.

Germination test of T_1 and T_2 generation embryos for bialaphos resistance

Immature embryos from T_0 and T_1 plants were examined for bialaphos resistance, by excising them from developing grains and culturing them on medium containing 3 mg l^{-1} bialaphos as described by Wan and Lemaux (1994).

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References

Bevan, M., Barnes, W.M. and Chilton, M. (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucl. Acids Res,* **11,** 369–385.

Bidney, D., Scelonge, C., Martich, J., Burrus, M., Sims, L. and Huffman, G. (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **18,** 301–313.

Boulton, M.L., Bucholz, W.G., Marks, M.S., Markham, P.G. and Davies, J.W. (1989) Specificity of *Agrobacterium*-mediated delivery of maize streak virus to members of the Graminae. *Plant Mol. Biol.* **12,** 31–40.

Chan, M.-T., Chang, H.-H., Ho, S.-L., Tong, W.-F. and Yu, S.-M. (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. *Plant Mol. Biol.* **22,** 491–506.

Chan, M.-T., Lee, T.-M. and Chang, H.-H. (1992) Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiol.* 33, 577–583.

Christensen, A.H., Sharrock, R.A. and Quail, P.H. (1992) Maize polyubiquitin genes: genes, structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* **18,** 675–689.

Creissen, G., Smith, C., Franic, R., Reynolds, H. and Mullineaux, P. (1990) *Agrobacterium*- and microprojectile-mediated viral DNA delivery into barley microspore-derived cultures. *Plant Cell Rep.* **8,** 680–683.

Czernilofsky, A.P., Hain, R., Herrera-Estrella, L., Lörz, H., Goyvaerts, E., Baker, B.J. and Schell, J. (1986) Fate of selectable marker DNA integrated into the genome of *Nicotiana tabacum*. *DNA* **5,** 101–113.

Deng, W.Y., Lin, X.Y. and Shao, Q.Q. (1990) *Agrobacterium tumefaciens* can transform *Triticum aestivum* and *Hordeum vulgare* of Gramineae. *Science in China (ser. B)* **33,** 27–34.

Engler-Blum, G., Meier, M., Frank, J. and Muller, G.A. (1993) Reduction of background problems in nonradioactive Northern and Southern Blot analyses enables higher sensitivity than ^{32}P-based hybridizations. *Anal. Biochem.* **210,** 235–244.

Funatsuki, H., Kuroda, H., Kihara, M., Lazzeri, P. A., Müller, E., Lörz, H. and Kishinami, I. (1995) Fertile transgenic barley generated by direct DNA transfer to protoplasts. *Theor. Appl. Genet.* **91,** 707–712.

Garfinkel, M. and Nester, E.W. (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumourigenesis and octopine catabolism. *J. Bacteriol.* **144,** 732–743.

Gould, J., Devery, M., Hasegawa, O., Ulian, E.C., Peterson, G. and Smith, R.H. (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol.* 95, 426–434.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6,** 271–282.

Hunter, C.P. (1988) Plant regeneration from microspores of barley, *Hordeum vulgare*. Ph.D. Thesis. Ashford: Wye College, University of London.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnol.* **14,** 745–750.

Jähne, A., Becker, D., Brettschneider, R. and Lörz, H. (1994) Regeneration of transgenic, microspore-derived, fertile barley. *Theor. Appl. Genet.* 89, 525–533.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Knittel, N., Gruber, V., Hahne, G and Lénée, P. (1994) Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. *Plant Cell Rep.* **14**, 81–86.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Körber, H., Redei, G.P. and Schell, J. (1989) High-frequency T-DNA-mediated gene tagging in plants. *Proc. Natl Acad. Sci. USA*, **86**, 8467–8471.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technology* **9**, 963–967.

Li, Z., Upadhyaya, N.M., Meena, S., Gibbs, A.J. and Waterhouse P.M. (1997) Comparison of promoters and selectable marker genes for use in Indica rice transformation. *Mol. Breeding*, **3**, 1–14.

McElroy, D., Blowers, A.D., Jenes, B. and Wu, R. (1991) Construction of expression vectors based on the rice actin 1 (*Act1*) 5′ region for use in monocot transformation. *Mol. Gen. Genet.* **231**, 150–160.

McElroy, D. and Jacobsen, J. (1995) What's brewing in barley biotechnology? *Bio/Technology* **13**, 245–249.

Martienssen, R.A., Barkan, A., Freeling, M. and Taylor, W.C. (1989) Molecular cloning of a maize gene involved in photosynthetic membrane organisation that is regulated by Robertson's Mutator. *EMBO J.* **8**, 1633–1639.

Mooney, P.A., Goodwin, P.B., Dennis, E.S. and Llewellyn, D.J. (1991) *Agrobacterium tumefaciens*-gene transfer into wheat tissues. *Plant Cell, Tissue Organ Culture* **25**, 209–218.

Potrykus, I. (1990) Gene transfer to cereals: an assessment. *Bio/Technology* **8**, 535–542.

Raineri, D.M., Bottino, P., Gordon, M.P. and Nester, E.W. (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). *Bio/Technology* **8**, 33–37

Rashid, H., Yokoi, S., Toriyama, K. and Hinata, K. (1996) Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. *Plant Cell Rep.* **15**, 727–730.

Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D. and Higgins, T.J.V. (1993) Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). *Plant Physiol.* **101**, 751–757.

Tabe, L.M., Wardley-Richardson, T., Ceriotti, A., Aryan, A., McNabb, W., Moore, A. and Higgins, T.J.V. (1995) A biotechnological approach to improving the nutritive value of alfalfa. *J Anim. Sci.* **73**, 2752–2759.

Thompson, C.J., Novva, N.R., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M. and Botterman, J. (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* **6**, 2519–2523.

Wan, Y. and Lemaux, P.G. (1994) Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol.* **104**, 37–48.

Xie, Y., Pen, Z., Cai, Y. and Wu, R. (1987) Cloning and analysis of histone 3 gene and rubisco small subunit gene of rice. *Sci. Sinica* (*ser B*) **30**, 706–718.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Ann. Rev. Genet.* **22**, 1–30.

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Transformation and regeneration capacities for five Nordic barley elite cultivars — evaluation of tissue culture response and transient expression

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Using both biolistic and *Agrobacterium*-mediated DNA delivery, we have investigated the transformation and regeneration capacity for five Nordic elite cultivars of barley. Transformation was followed as transient expression of the *uid*A or *gfp* gene in barley callus. Callus formation and regeneration of transformed callus were evaluated based on callus induction frequency, growth rate, callus appearance, and shoot formation frequency. From the accumulated results, one of the elite cultivars has been selected for our ongoing work in molecular breeding of barley.

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Efficient genetic improvement of commercial crop varieties by molecular breeding techniques requires novel traits be introduced into local elite cultivars. Introduction of genes using genetic engineering is dependent on efficient and reproducible in vitro protocols. Based on tissue culture protocols for dicotyledonous plants, several modifications have been introduced to improve culture conditions for monocotyledons; e.g. addition of copper and BAP in callus induction medium (CHO et al. 1998), culture induction under dim light or in darkness (ALTPETER et al. 1996; CHO et al. 1999), and efficient selection pressure determined by the CPR assay (HÄNSCH et al. 1998).

Fertile transgenic barley plants can be routinely produced by microprojectile bombardment of explant material (WAN and LEMAUX 1994). However, its efficiency is still low, and it is often associated with multiple integration events or extensive DNA rearrangements. To overcome these problems, *Agrobacterium*-mediated T-DNA transfer can be an alternative. Recently, transgenic plants from barley were obtained by *Agrobacterium*-mediated transformation (TINGAY et al. 1997; PATEL et al. 2000).

In most reports on transgenic barley, the genotype Golden Promise (GP) has been used. KOPREK et al. (1996) and JIANG et al. (1998) have reported that immature embryos for most commercially important barley cultivars have a low callus induction rate. Thus transformation protocols developed for a model cultivar such as GP need to be adjusted and optimized for commercial cultivars and breeding lines before use (CHO et al. 1998).

The purpose of the present work was to investigate regeneration and transformation capacities of five Nordic elite barley cultivars (Baronesse, Cecilia, Filippa, Mentor and Pongo). We studied the callus-induction frequency, growth rate, callus quality and shoot formation frequency. Both methods of transformation (biolistic and *Agrobacterium*-based) were used to evaluate their transformation capacity using both GUS and GFP as reporter systems.

MATERIALS AND METHODS

Plant material

Barley plants (*Hordeum vulgare* L. cv. Baronesse, Cecilia, Filippa, Mentor and Pongo) were grown in growth chambers under a 16-h light/8-h dark period at 18°C and 60–80 % humidity as described by WAN and LEMAUX (1994).

Callus culture

Callus cultures were established according to AHLANDSBERG et al. (1999). The callus induction medium (CIM) was a MURASHIGE and SKOOG (1962) basal medium supplemented with 30 g/l maltose, 1.0 mg/l thiamine-HCl, 0.25 g/l myo-inositol, 1.0 g/l casein hydrosylate, 0.69 g/l proline, 5 µM cupric sulfate and 2.5 mg/l dicamba solidified by 5.0 g/l Gelrite.

Callus induction frequency, growth rate, callus quality and shoot formation

Immature embryos (IEs) of about 1.0–2.5 mm were isolated from seeds and surface-sterilized for 15 min

in 20 % bleach (v/v) followed by three washes in sterile water. IEs, scutellum-side down, were grown on CIM. Embryos were incubated at 24°C in the dark. Callus induction frequency was measured by counting IEs producing callus 3 weeks after plating. To determine growth rate, 10 IEs were cultured on plate with three replicates for each cultivar. The initial callus growth rate was determined by weighing the 10 IEs at time zero (W1) and 21 days after initial callus induction (W2). Growth rate was calculated as the change in tissue weight (W1–W2) divided by the number of embryos plated and days of culturing (mg embryo^{-1} day^{-1}). Callus quality was assessed three weeks after initial callus induction. A score of + + + + + was given to shiny, compact, nodular slightly brown-colored callus (the highest quality), a score of + being given to soft, friable, white callus (the lowest quality). After 3 weeks, callus were transferred to light (65–100 μE m^{-2} s^{-1}) and the shoot formation frequency was measured after another 2–3 weeks by counting the number of shoot-producing callus. In addition, the number of shoots per callus was detected.

Agrobacterium strain and plasmids

Agrobacterium tumefaciens AGL1 (LAZO et al. 1991) was used in the transformation experiments. Plasmid pDM805 (TINGAY et al. 1997) contains, between the T-DNA borders, a chimeric *Streptomyces hygroscopicus* phosphinothricin acetyl transferase (bar) gene (THOMPSON et al. 1987) under control of the intronic promoter of the maize ubiquitin 1 (*Ubi1*) gene (CHRISTENSEN et al. 1992) and a transcription terminator sequence from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene (BEVAN et al. 1983). It also harbors a chimeric *E. coli* β-glucuronidase (*uidA*) gene (JEFFERSON et al. 1987), under control of the intronic promoter of the rice actin 1 (*Act1*) gene (MCELROY et al. 1991) and a transcription terminator sequence from the rice *rbcS* gene (XIE et al. 1987), encoding the small subunit of ribulose bis-phosphate carboxylase/oxygenase (Fig. 1A).

The plasmid pN1473GFP, used for biolistic transformations, contains the *gfp* gene (CHALFIE et al. 1994), encoding the green fluorescent protein (GFP), under control of the *Act1* promoter and *nos* terminator (AHLANDSBERG et al. 1999) (Fig. 1B).

Transformation

Agrobacterium tumefaciens AGL1 (pDM805) was grown from a single colony in LB medium (MILLER 1972) supplemented with 20 mg/l rifampicin and 5 mg/l tetracycline for 2 days at 27°C. Colonies were resuspended in MSMG liquid medium (basal MS medium with 2 g/l glucose, 0.01 % pluronic F68 (Sigma) and 200 μM acetosyringone (AS)) for 3 h. The final concentration was adjusted to 1×10^9 bacterial cell ml^{-1}. Transformation procedures were mainly according to TINGAY et al. (1997). One day after isolation, IEs were immersed in full-strength *Agrobacterium* suspension medium and immediately transferred, without rinsing, to co-cultivation medium (callus induction medium with 200 μM acetosyringone), with the scutellar surface placed in contact with the medium. Plates were incubated at 24°C in darkness for 3 days. After co-cultivation, embryos were transferred directly to CIM supplemented with 3 mg/l bialaphos and 150 mg/l Timentin TM (Smithkline Beecham, England). The selection process proceeded for up to 3 weeks.

Microprojectile bombardment was done according to WAN and LEMAUX (1994) using a Du-Pont PDS-1000 He Biolistic delivery System (Bio-Rad Laboratories, USA). Gold particles were coated with DNA using the procedure of DAINES (1990). Four hours prior to bombardment, embryos were placed on osmotic treatment plates (CIM medium supplemented with 27 g/l mannitol) and positioned scutellum side up. Fifty half-IEs were placed in the center of a Petri dish and bombarded. Embryos were kept on osmotic treatment plates 16 h after bombardment and were then transferred to CIM plates with the scutellum side down.

Chlorophenol-red assay (CPR assay)

The CPR assay was performed according to HÄNSCH et al. (1998). IEs were incubated for 2 days on CIM medium containing 50 mg/l CPR (Sigma) and different concentrations of selective agent bialaphos (0, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/l). The pH of the medium was adjusted to 6.0, a pH at which CPR shows a deep red color. Both CPR and the selective agent

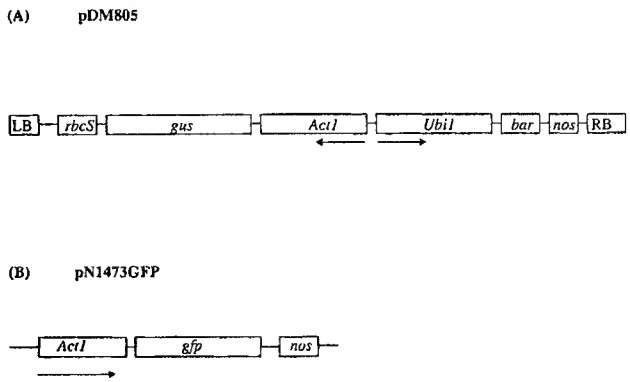

Fig. 1 A and B. Structure and restriction map of the barley transformation vectors pDM805 **(A)** and pN1473GFP **(B)**. The direction for transcription of the *Act1* and *Ubil* genes is indicated by arrows. The vector elements are not drawn to scale.

Table 1. *Callus induction frequency, initial growth rate, callus quality and shoot formation of five barley elite cultivars (Baronesse, Cecilia, Filippa, Mentor and Pongo)*[a]

Cultivars	Callus induction frequency[a] (%)	Initial callus growth rate (mg embryo^{-1} day^{-1})	Callus quality	Shoot formation frequency[a] (%)	Number of shoots per callus
Baronesse	100 ± 0^{b}	2.7 ± 0.3	++	36.7 ± 12.5^{e}	1.1 ± 0.1
Cecilia	90 ± 10^{f}	3.0 ± 0.2	+++	$64 \pm 19.4^{c,d}$	1.3 ± 0.2
Filippa	100 ± 0^{b}	2.9 ± 0.2	++++	$40 \pm 28.3^{d,e}$	1.0 ± 0
Mento	100 ± 0^{b}	1.6 ± 0	+	$50 \pm 37^{c,d,e}$	1.2 ± 0.2
Pongo	100 ± 0^{b}	1.8 ± 0.1	++++	70 ± 21.6^{c}	1.8 ± 0.8

[a] Percentages with a different letter (b–e) are significantly different at $p = 0.05$. Three replicates of 10 IEs for each cultivar were transferred to CIM. Values for callus-induction frequency and initial callus growth rate were measured 24 days after the initial callus induction. Callus quality was assessed microscopically and scored on a scale with ++++ being the highest quality and + being the lowest. Callus were transferred to light and shoot formation frequency and number of shoots produced per callus were recorded after 2–3 weeks.

were filter-sterilized and added after autoclaving. To keep conditions comparable, 10 IEs were used in all cases and placed on 3.5-cm Petri dishes containing 1.6 ml medium. After 2 days incubation in the dark at 25°C, visible color changes occurred. The solid medium as well as the explants were transferred to 2-ml Eppendorf tubes and centrifuged for 30 min at 15,000 g. For the assay, 400 µl of the supernatant was removed, diluted 1:1 with water and measured at 575 nm (the absorption maximum of CPR at pH 6.2). Three replicates were performed for each treatment.

Enzyme assays

GUS activity was assayed histochemically as described by HÄNSCH et al. (1995). The explants were pretreated for 60 min at 55°C followed by staining at the same temperature to suppress barley endogenous β-glucuronidase activity. An explant was scored as GUS-positive based on the presence of any blue cells in IEs or callus. Transient expression of the *uidA* gene was assayed 3 and 21 days after inoculation (*Agrobacterium*-mediated transformation) for all the genotypes tested. Every experiment was conducted and confirmed at least three times.

In situ assays of *gfp* expression were done according to AHLANDSBERG et al. (1999) using an epifluorescent stereo microscope, equipped with a double set of fluorescence filters (excitation 480 ± 40 nm; dicroic 505 LP nm and 510 LP nm). The *gfp* expression was assayed 3 days after biolistic transformation. Three replicates of 50 IEs each were carried out.

RESULTS AND DISCUSSION

Induction of callus and shoot formation

All genotypes tested produced callus on CIM medium with a high callus frequency, more than 90 % (Table 1). The growth rate of callus was between 1.6 mg embryo^{-1} day^{-1} (Mentor) and 3.0 mg embryo^{-1} day^{-1} (Cecilia). This latter result was different from JARL (1999) who reported that Mentor had a high growth rate. The reason for this discrepancy is not obvious since there is very little information provided in the report by JARL (1999) regarding the tissue culture and transformation procedures. In our opinion, the two most relevant differences between the five barley elite cultivars tested were the callus quality and the shoot formation frequency. Pongo and Filippa produced the highest quality of callus; shiny, compact, nodular, brown-colored callus. After transfer to light, shoots appeared on callus from all genotypes after 3 weeks. As is clear from Table 1, shoot production differed between genotypes; Pongo showed the best results in terms of shoot formation frequency and number of shoot produced per callus.

Selection

An efficient selection system is an important parameter in order to select a small number of stably transformed cells, especially when multicellular targets like IEs or suspension-derived embryos are used. Preliminary experiments are often required to determine a dose-response curve and to evaluate the minimal concentration required for efficient selection under established culture conditions. By using too high concentration of the selective agent, transformants with a high copy number are preferentially recovered and, also, the albinism problem is exacerbated (DALTON et al. 1995; LEMAUX et al. 1996).

Addition of the pH indicator chlorophenol-red to CIM medium resulted in a deep red color. After 2 days, a clear yellow color was visible indicating a decrease in the pH resulting from cell activity and ammonium accumulation. Spectrophotometric analysis was used to quantify the interaction between bialaphos concentration and cultivar (Fig. 2). HÄNSCH et al. (1998) reported that the amount of bialaphos required for a given genotype to yield an

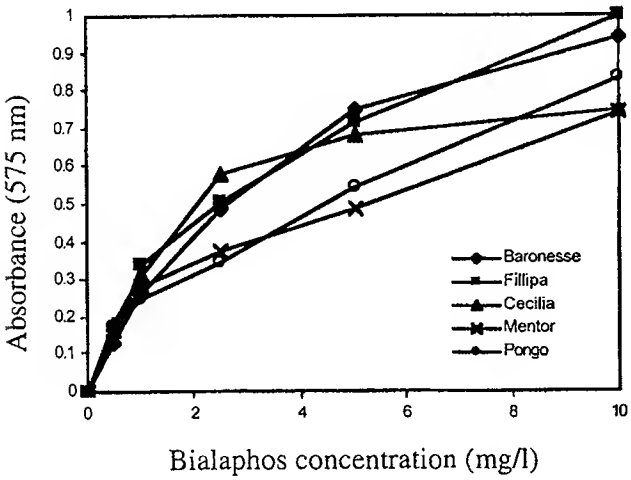

Fig. 2. Effect of bialaphos concentration (0–10 mg/l) on the absorbance of the clear supernatant from CIM containing CPR two days after inoculation of 10 IEs per plate for different barley genotypes. Each point is the mean of three independent replications.

absorbance of 0.5, represents a tight selection condition for the appropriate genotype. Embryos of the genotype Cecilia showed a very high response to the selective agent; a concentration of 2 mg/l of bialaphos was enough to prevent the color change. In contrast, embryos of Mentor gave a relatively low response (5 mg/l). The degree of color change at a given bialaphos concentration was a function of the genotype. These observations are consistent with the conclusion of HÄNSCH et al. (1998).

Transformation

TINGAY et al. (1997) were the first to report *Agrobacterium*-mediated transformation of barley. In their work, they found that wounding the IEs with a shot of gold particles enhanced the recovery of transformed tissue. In our case, we wanted to explore the possibility to avoid the biolistic step in the *Agrobacterium*-mediated transformation system. To this end, we adopted the protocol by TINGAY et al. (1997) with the following modifications: (I) bacteria were induced 3 h with acetosyringone (200 µM) (GUO et al. 1998) to induce the *vir* genes; (II) Pluronic F68 (0.01 %) (CHENG et al. 1997) was present in the inoculation medium; (III) IES were co-cultivated on CIM medium with 200 µM of acetosyringone.

The percentage of immature embryos expressing the *uidA* gene varied between the different genotype (Table 2); Baronesse and Mentor gave the highest frequencies of expression, while Cecilia, Filippa and Pongo showed lower frequencies of expression (Table 2). After 3 weeks on selective CIM medium (3–5 mg/l of bialaphos), IEs were sampled for GUS assays. All the cultivars tested showed stable expression of the reporter gene (Table 2). There was no significant difference between them at $p = 0.05$. Therefore, stable expression of the *gus* gene can be obtained without biolistic wounding of the IEs.

AHLANDSBERG et al. (1999) have reported that the *gfp* gene can be used as a reporter gene for barley transformation. All the genotypes tested were expressing the *gfp* after biolistic transformation (Table 2); the highest expressions were found in Mentor and Pongo, while Cecilia showed the lowest level of expression. The differences in results for the two transformation systems might be explained by the fact that cells need to be "competent" for transformation by *Agrobacterium tumefaciens*. Indeed, SANGWAN et al. (1992) and VILLEMONT et al. (1997) showed that plant cell division and proliferation are required for stable expression of foreign genes following *Agrobacterium*-mediated transformation.

A comparative analysis of the results in Tables 1 and 2 reveals that the general amenableness of a crop plant to genetic engineering is a complex mixture of several parameters. All of the tested barley cultivars are to some extent suitable for transformation with

Table 2. *Percentage of IEs expressing the uidA or gfp gene*[a]

Cultivar	*Agrobacterium*-mediated transformation (% explants expressing *uidA*)[a]		Microprojectile bombardment (% of explants expressing *gfp*)[a]
	3 days	21 days	3 days
Control	0	0	0
Baronesse	30 ± 17.6^{b}	19 ± 6.2^{d}	$37 \pm 12.5^{e,f}$
Cecilia	8 ± 7.7^{c}	10 ± 8^{d}	17 ± 7.2^{e}
Filippa	9 ± 8.4^{c}	14 ± 5^{d}	$32 \pm 8.3^{e,f}$
Mentor	$15 \pm 12.2^{b,c}$	4 ± 3.8^{d}	$43 \pm 7.2^{e,f}$
Pongo	11 ± 3.4^{c}	10 ± 9^{d}	50 ± 1.6^{f}

[a] Percentages with a different letter (b–f) are significantly different at $p = 0.05$. Results were scored by the GUS assay 3 or 21 days after inoculation for *Agrobacterium*-mediated transformation and by fluorescent foci 3 days after biolistic transformation.

either or both of the *Agrobacterium*-based or biolistic techniques. The overall performance was best for cultivars Pongo, Baronesse and Filippa. No single cultivar stood out as superior in terms of transformation ability. Of the parameters listed in Table 1, we consider callus quality and shoot formation to be the most important criteria. Based on these considerations, we selected the elite cultivar Pongo for our future experiments on molecular breeding of barley.

ACKNOWLEDGEMENTS

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REFERENCES

Ahlandsberg S, Sathish P, Sun C and Jansson C, (1999). Green fluorescent protein as a reporter system in the transformation of barley cultivar. Physiol. Plant. 107: 194–200.

Altpeter F, Vasil V, Srivastava V, Stöger E and Vasil IK, (1996). Accelerated production of transgenic wheat (Triticum aestivum L.) plants. Plant. Cell Rep. 16: 12–17.

Bevan M, Barnes WM and Chilton M, (1983). Structure and transcription of the nopaline synthase gene region of T-DNA. Nucl. Acids Res. 11: 369–385.

Chalfie M, Tu Y, Euskirchen G, Ward WW and Prasher DC, (1994). Green fluorescent protein as a marker for gene expression. Science 263: 802–805.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW and Wan Y, (1997). Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant. Physiol. 115: 971–980.

Cho M-J, Jiang W and Lemaux PG, (1998). Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. Plant Science 138: 229–244.

Cho M-J, Jiang W and Lemaux PG, (1999). High-frequency transformation of oat via microprojectile bombardment of seed-derived highly regenerative cultures. Plant Science 148: 9–17.

Christensen AH, Sharrock RA and Quail PH (1992). Maize polyubiquitin genes: genes, structure thermal pertubation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plan. Mol. Biol. 18: 675–689.

Daines RJ, (1990). Dekalb researchers improve maize transformation rates. Biolistic Syst. Newslett. 1: 1–4.

Dalton SJ, Bettany AJE, Timms E and Morris P, (1995). The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (Festuca arundinacea Schreb.). Plant Sci. 108: 63–70.

Guo GQ, Maiwald F, Lorenzen P and Steinbiss HH, (1998). Factors influencing T-DNA transfer into wheat and barley cells by Agrobacterium tumefaciens. Cereal Res. Commun. 26: 15–22.

Hänsch R, Koprek T, Mendel RR and Schuize J, (1995). An improved protocol for eliminating endogenous β-glucuronidase background in barley. Plant Sci. 105: 63–69.

Hänsch R, Mendel RR and Schulze J, (1998). A rapid and sensitive method to evaluate genotype specific tolerance to phosphinothricin-based selective agents in cereal transformation. J. Plant Physiol. 52: 145–150.

Jarl CI, (1999). Plant regeneration and transient expression after particle bombardment of different barley (Hordeum vulgare L.) genotypes. Hereditas 130: 83–87.

Jefferson RA, Kavanagh TA and Bevan MW, (1987). GUS fusion: β-glucuromdase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Jiang W, Cho M-J and Lemaux PG, (1998). Improved callus quality and prolonged regenerability in model and recalcitrant barley (Hordeum vulgare L.) cultivars. Plant Biotechnol. 15: 63–69.

Koprek T, Hänsch R, Nerlich A, Mendel RR and Schulze J, (1996). Fertile transgenic barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. Plant Sci. 19: 79–91.

Lazo GR, Stein PA and Ludwig RA, (1991). A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Bio/Technology 9: 963–967.

Lemaux PG, Cho MJ, Louwerse J, Williams R and Wan Y, (1996). Bombardment-mediated transformation method for barley. Biorad US/EG Bulletin 2007.

McElroy D, Blowers AD, Jenes B and Wu R, (1991). Construction of expression vectors based on the rice actin 1 (Act1) 5′ region for use in monocot transformation. Mol. Gen. Genet. 231: 150–160.

Miller JH, (1972). In: Experiments in molecular genetics. Cold Spring Harbor, New York.

Murashige T and Skoog F, (1962). A revised medium for rapid growth and bioassays tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Patel M, Johnson JS, Brettell RIS, Jacobsen J and Xue G-P, (2000). Transgenic barley expressing a fungal xylase gene in the endosperm of the developing grains. Mol. Breed. 6: 113–123.

Sangwan RS, Bourgeois Y, Brown S, Vasseur G and Sangwan-Norrel BS, (1992). Characterization of competent cells and early events Agrobacterium-mediated genetic transformation in Arabidopsis thaliana. Planta 188: 439–456.

Thompson CJ, Novva NR, Tizard R, Crameri R, Davies JE, Lauwereys M and Botterman J, (1987). Characterization of the herbicide-resistance gene bar from Streptomyces hygroscopicus. EMBO J. 6: 2519–2523.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell R, (1997). Agrobacterium tumefaciens-mediated barley transformation. Plant J. 11: 1369–1376.

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y and Sangwan-Norrel BS, (1997). Role of the host cell cycle in the Agrobacterium-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160–172.

Wan Y and Lemaux PG, (1994). Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37–48.

Xie Y, Pen Z, Cai Y and Wu R, (1987). Cloning and analysis of histone 3 gene and rubisco small subunit gene of rice. Sci. Sinica (ser B) 30: 706–718.

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TECHNICAL ADVANCE

Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA

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Summary

A large number of morphologically normal, fertile, transgenic rice plants were obtained by co-cultivation of rice tissues with *Agrobacterium tumefaciens*. The efficiency of transformation was similar to that obtained by the methods used routinely for transformation of dicotyledons with the bacterium. Stable integration, expression and inheritance of transgenes were demonstrated by molecular and genetic analysis of transformants in the R_0, R_1 and R_2 generations. Sequence analysis revealed that the boundaries of the T-DNA in transgenic rice plants were essentially identical to those in transgenic dicotyledons. Calli induced from scutella were very good starting materials. A strain of *A. tumefaciens* that carried a so-called 'super-binary' vector gave especially high frequencies of transformation of various cultivars of japonica rice that included Koshihikari, which normally shows poor responses in tissue culture.

Introduction

Methods for transformation of higher plants employing *Agrobacterium* have been well established for dicotyledonous species but not for monocotyledonous species, except in a few cases (Bytebier *et al.*, 1987). Transformation of plants in Gramineae with *Agrobacterium*, including *Agrobacterium*-mediated infection of plants with viral genomes, has been attempted in several laboratories (Chan *et al.*, 1992, 1993; Gould *et al.*, 1991; Grimsley *et al.*, 1988; Mooney *et al.*, 1991; Raineri *et al.*, 1990; Schläppi and Hohn 1992; Shen *et al.*, 1993). Raineri *et al.* (1990) obtained transformed rice cells that expressed neomycin-phosphotransferase (NPT) and β-glucuronidase (GUS) activities and they suggested that T-DNA had been transferred to, integrated in, and expressed in, rice cells. Gould *et al.* (1991) described the transfer of genes for NPT and GUS into shoot apices of corn, subsequent regeneration of plants, and detection of the transferred genes in the F_1 progeny by Southern hybridization.

These early studies of *Agrobacterium*-mediated transformation of monocotyledons have, however, been controversial. For example, Potrykus (1990) presented a critical review of the cited reports and suggested that the various authors might have overlooked the possibility of gene expression by *Agrobacterium* attached to inoculated tissues and to the plantlets regenerated from them, as well as transformation of microorganisms that were silently infecting the host plant tissues. Potrykus concluded that there was no unequivocal evidence for stable transformation of monocotyledons with *Agrobacterium*.

Appropriate evidence for such transformation would be a demonstration of random integration of transgenes into chromosomes in a number of independent transformants, with Mendelian segregation of transgenes in the progeny. Recently, Chan *et al.* (1993) obtained a few transgenic rice plants by inoculating immature embryos with a strain of *A. tumefaciens*. They proved the inheritance of the transferred DNA to the progeny by Southern hybridization, although the progeny of only one plant was analysed.

We now present further evidence in an attempt to resolve the controversy. Our evidence is based on molecular and genetic studies of a large number of transgenic rice plants and the analysis of T-DNA junctions in rice. We also show that co-cultivation of calli, derived from scutella, with *A. tumefaciens* can produce rice transformants with an efficiency similar to that of transformation in dicotyledons.

Results

Production of hygromycin-resistant, GUS-expressing plants from rice tissues inoculated with A. tumefaciens

Various tissues from rice, namely, shoot apices and segments of roots from young seedlings, scutella, immature embryos, calli induced from young roots and scutella, and cells in suspension cultures induced from scutella, were

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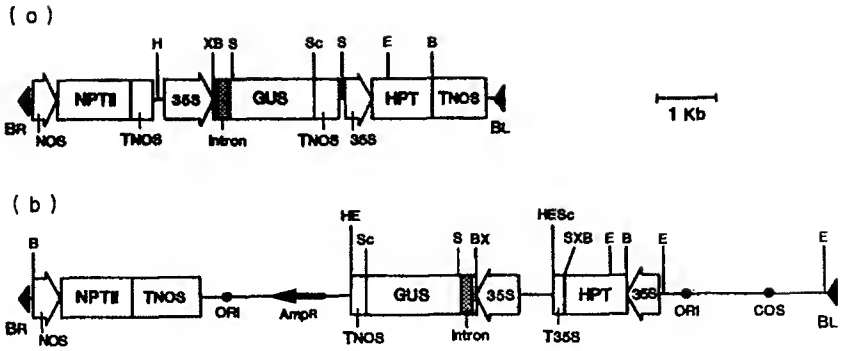

Figure 1. T-DNA regions of pIG121Hm (a) and pTOK233 (b).
Abbreviations: BR, right border; BL, left border; NPTII, neomycin phosphotransferase; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; NOS, nopaline synthase promoter; 35S, 35S promoter; TNOS, 3' signal of nopaline synthase; T35S, 3' signal of 35S RNA; ORI, origin of replication of ColE1; AmpR, ampicillin-resistance gene active in *E. coli*; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sc, *Sac*I; X, *Xba*I.

Table 1. Expression of GUS in rice tissues (cv. Tsukinohikari) immediately after co-cultivation with *A. tumefaciens* EHA101(pIG121Hm) and the recovery of GUS-expressing (GUS+), hygromycin-resistant (HygR) cells

		Number of pieces of tissue					
		Immediately after co-cultivation			After 3 weeks on selective medium		
Tissue	Duration of co-cultivation (days)	GUS+	Co-cultivated	(%)	Produced GUS+, HygR cells	Co-cultivated	(%)
Shoot apex	2	40	80	(50)			
Shoot apex	5	69	77	(90)	0	77	(0)
Root segment	3	0	100	(0)			
Scutellum	3	8	89	(9)	0	128	(0)
Immature embryo	3	66	198	(33)	3	51	(6)
Root callus	3	24	115	(21)			
Scutellum callus	3	497	533	(93)	169	743	(23)
Suspension cells[a]	3	61	247	(25)	22	254	(9)

[a]Cluster of 1–2 mm in diameter was counted as a piece of tissue.

co-cultivated with *A. tumefaciens* EHA101(pIG121Hm) (Figure 1) and GUS expression was examined immediately (Figure 2a, Table 1). Expression of GUS was detected in all of the tissues examined apart from the root segments. The calli derived from scutella gave the highest ratio of GUS-expressing tissue to inoculated tissue, and GUS expression in immature embryos was observed mainly in scutella.

Rice tissues that had been co-cultivated with *A. tumefaciens* EHA101(pIG121Hm) were cultured on hygromycin-containing medium. Proliferation of cells on the selective medium was observed in the case of the calli induced from scutella (Figure 2b), immature embryos and cells in suspension culture (Table 1). The calli gave the highest frequency (23%; 169/743) of tissue pieces that produced hygromycin-resistant cells. The proliferating regions showed evidence of the uniform expression of GUS (Figure 2c).

Calli derived from scutella were co-cultivated with four strains of *A. tumefaciens* and then cultured on selective medium. LBA4404(pTOK233) (Figure 1) gave the highest frequency of callus pieces that produced hygromycin-resistant cells (Table 2). The superiority of LBA4404(pTOK233) was especially evident in the case of cv. Koshihikari.

Most of the colonies of cells that recovered from the first round of selection proliferated on the second selective medium. Plants were readily regenerated when the resistant cells were transferred to a regeneration medium that contained hygromycin (Figure 2d). The frequency of regeneration ranged from 50 to 80% of selected colonies. Hundreds of hygromycin-resistant plants that stably expressed GUS activity were obtained. The most efficient strain, LBA4404(pTOK233), produced such plants from all of the cultivars tested, at frequencies between 12.8

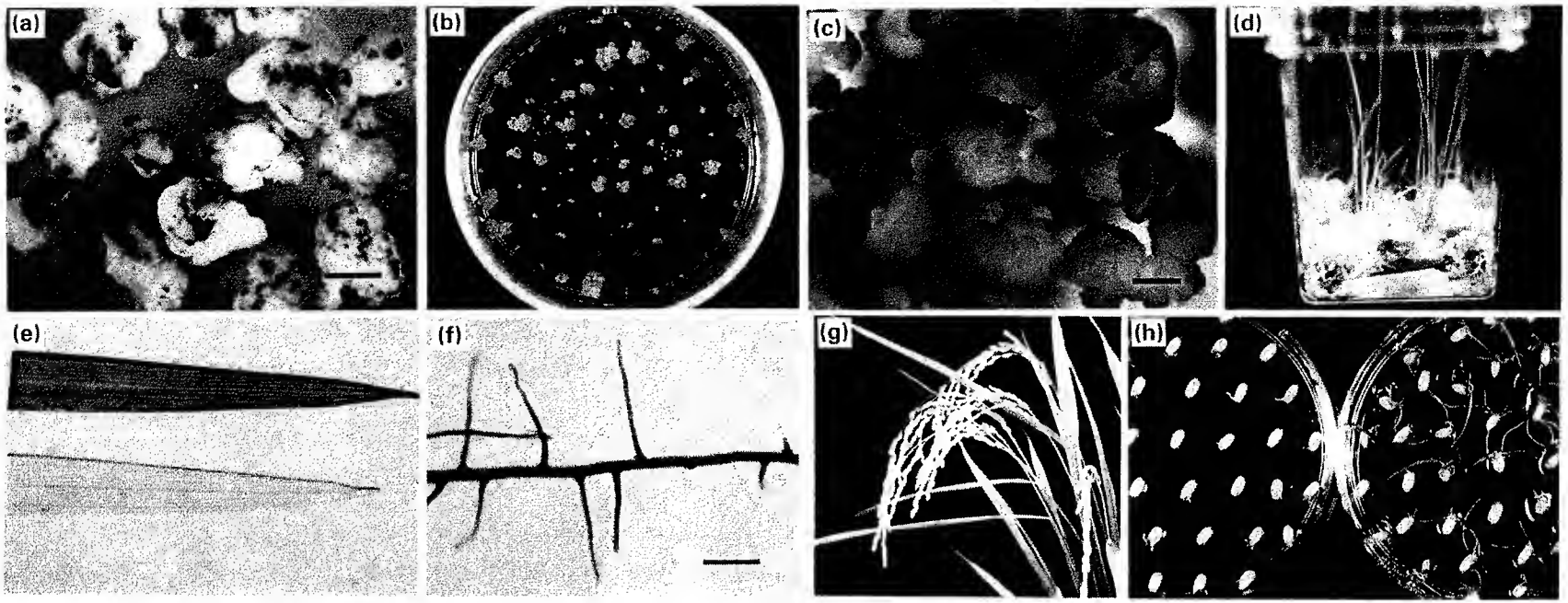

Figure 2. Scutellum-derived calli of rice cv. Tsukinohikari that had been co-cultivated with *A. tumefaciens* EHA101(pIG121Hm), and transgenic cells and plants derived from the calli.
(a) Expression of GUS after infection. The calli were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) after 3 days of co-cultivation.
(b) Hygromycin-resistant colonies of cells. The calli were plated on a selective medium after co-cultivation. The photograph was taken after 3 weeks of selection.
(c) Expression of GUS in hygromycin-resistant colonies of cells. Colonies of cells proliferated from the calli were stained with X-Gluc after 3 weeks of selection.
(d) Plant regeneration. The photograph was taken 4 weeks after selected cells had been transferred to regeneration medium.
(e) Expression of GUS in the leaf of a transformant. Segments of leaves of a transgenic plant (upper) and a non-transgenic plant (lower) were stained with X-Gluc.
(f) Expression of GUS in the root of a transformant. A segment of the root of a transgenic plant was stained with X-Gluc.
(g) A transformant at maturity.
(h) Test of the progeny for resistance to hygromycin. Seeds from a non-transgenic (left) and a transgenic (right) plant were plated on selective medium and the photograph was taken 3 days later.
Bars in the photographs represents 1 mm.

Table 2. Production of GUS-expressing (GUS+), hygromycin-resistant (HygR) cells from scutellum-derived calli inoculated with various strains of *A. tumefaciens*

Rice cultivar	Experiment	Calli that produced GUS+, HygR cells / calli inoculated (%)			
		LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK233)	EHA101 (pTOK233)
Tsukinohikari	1		10/521 (2)	141/540 (26)	22/605 (4)
	2		56/187 (30)	114/324 (35)	44/254 (17)
	3		20/325 (6)	100/349 (29)	3/314 (1)
	4	91/338 (27)	139/301 (46)	159/305 (52)	
	5	59/421 (14)	66/425 (16)	110/360 (31)	
	6	64/295 (22)	66/251 (26)	108/263 (41)	30/260 (12)
	7	63/436 (14)	172/394 (44)	229/397 (58)	45/420 (11)
	8	61/236 (26)	65/241 (27)	65/143 (45)	27/219 (12)
Koshihikari	1	6/269 (2)	7/271 (3)	65/283 (23)	
	2	3/262 (1)	18/310 (6)	60/273 (22)	3/251 (1)
	3	5/197 (3)	11/176 (6)	34/138 (25)	
	4	2/177 (1)	16/140 (11)	49/215 (23)	3/162 (2)

Table 3. Efficiency of rice transformation by *A. tumefaciens* LBA4404(pTOK233)

Rice cultivar	Experiment	Number of scutellum-derived calli				
		Inoculated (*A*)	Produced HygR cells	Produced HygR plants	Produced HygR and GUS+ plants (*B*)	Frequency (*B*/*A*, %)
Tsukinohikari	1	42	20	15	12	28.6
	2	112	35	19	15	13.4
	3	64	48	24	18	28.1
	4	65	29	23	13	20.0
	5	45	18	10	7	15.6
	6	88	24	21	16	18.2
Asanohikari	1	86	19	11	11	12.8
	2	77	23	17	15	19.5
Koshihikari	1	74	16	13	13	17.6
	2	138	34	26	26	18.8
	3	215	49	40	40	18.6

HygR, hygromycin-resistant.
GUS+, GUS-positive.

and 28.6% relative to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells (Table 3).

Many of the hygromycin-resistant cells showed uniform blue staining in the histochemical assay for GUS. However, some of the colonies of cells included unstained areas which suggested that some of the hygromycin-resistant colonies were chimeric. GUS assays of segments of leaves or roots from regenerated plants resulted in blue staining of entire samples in most cases (Figure 2e and f), but chimerism (periclinal chimeras and dot-like chimeras) was occasionally observed.

Characterization of the plants in the R_0 generation

Hygromycin-resistant, GUS-expressing plants were grown in a greenhouse, and evaluated for ploidy, morphology and fertility. A total of 103 plants (cv. Tsukinohikari) from co-cultivation of scutellum-derived calli with EHA101(pIG121Hm) and 196 plants (93 of cv. Tsukino-

hikari and 103 of cv. Koshihikari) from that with LBA4404(pTOK233) were examined. All of the plants were diploid, as revealed by flow-cytometric analysis (data not shown), and none of them exhibited morphological aberrations. In terms of fertility, by contrast, variations from complete sterility to full fertility were observed. Nevertheless, the majority (about 70%) of transformants produced as many seeds as seed-derived control plants (Figure 2g). The progeny of semi-fertile transformants are now under evaluation for fertility, and preliminary data indicate that they are full fertile.

Hygromycin-resistant, GUS-expressing plants were analyzed by Southern hybridization. For example, DNA from 29 plants derived from 20 independent colonies of resistant cells of cv. Tsukinohikari, which were produced by co-cultivation of scutellum-derived calli with EHA101(pIG121Hm), was digested with *Hin*dIII and allowed to hybridize with the *hpt* and *gus* probes (some of the resultant data are shown in Figure 3a and b). Since the T-DNA of pIG121Hm has a single *Hin*dIII site (Figure 1), the number of hybridizing bands reflected the number of copies of integrated genes in the plants unless repeats of multiple copies of the T-DNA had been integrated. Most of the detected bands represented fragments of more than 6.0 kb, which is the minimum size of hybridizing fragments expected from the map of pIG121Hm (Figure 1). Plants regenerated from a given colony of cells gave an identical pattern, indicating that these plants were clonal. Otherwise, the mobilities of the bands differed from plant to plant. The copy number of integrated genes varied from one to six (Table 4). Several

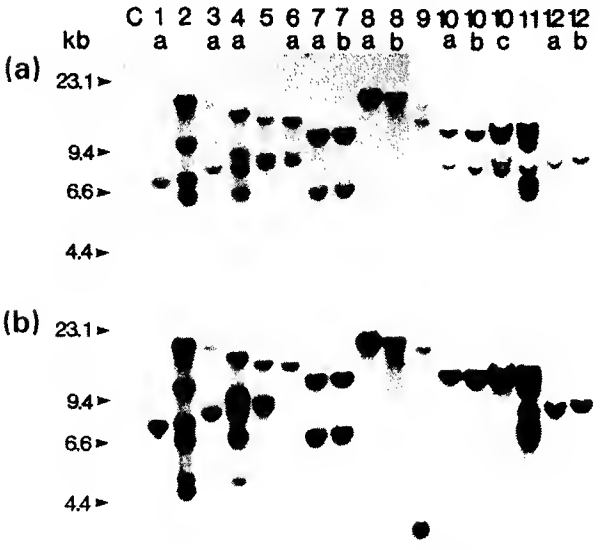

Figure 3. Southern analysis of transformants (R_0 generation).
DNA from a non-transgenic plant (C) and transformants (1a 12b) was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *hpt* (a) or the *gus* (b) probe.

plants gave signals only with the *hpt* probe, a few others gave signals only with the *gus* probe, and some of the fragments that hybridized with the *gus* probe were smaller than 6.0 kb (data not shown). These unexpected DNA fragments were probably produced by rearrangement of DNA upon transformation.

Inheritance of the marker genes

The selfed progeny were evaluated for resistance to hygromycin and GUS expression. The patterns of segregation of the offspring of 39 plants from the 20 independent colonies of cells described above are shown in Table 4. Resistant and sensitive seedlings were clearly distinguishable on agar plates that contained hygromycin (Figure 2h), and a segregation ratio of 3:1 was observed for 12 out of the 20 independent clones. The majority of the progeny showed clear segregation of GUS-positive and GUS-negative plants, and the estimated numbers of loci were consistent with those estimated on the basis of resistance to hygromycin.

The estimated numbers of loci were smaller than the numbers of copies of the genes that were measured by Southern analysis in some plants. It is likely that more than two copies of genes were integrated close to one another on a chromosome of such a plant. A few plants showed strange segregation patterns (plants 11, 18a, and 19e). For example, the number of hygromycin-resistant plants was smaller than that of sensitive plants in the progeny of plant 19e. Plants that gave unusual segregation ratios for hygromycin resistance gave similar results for GUS activity. Furthermore, plants that were chimeric for GUS activity were observed among such plants (progeny of plants 11, 18a, and 19e).

The R_1 progeny of plants 1b, 3a, 8a, 9, 10c and 12a, which gave segregation patterns of 3:1 or 15:1 for both hygromycin resistance and GUS expression, were analysed by Southern hybridization (parts of the data are shown in Figure 4). All of the hygromycin-resistant, GUS-positive progeny generated bands that hybridized with the *hpt* and *gus* probes whereas all of the sensitive, negative progeny did not.

Clear Mendelian segregation for hygromycin resistance and for GUS expression was also observed in the R_2 progeny of plants 1a, 3a, 8a, 9, 10c and 12a. For example, the R_2 progeny of two-thirds of the hygromycin-resistant, GUS-positive R_1 plants from plant 10c were hygromycin-resistant, GUS-positive plants and hygromycin-sensitive, GUS-negative plants at a ratio of 3:1. The R_2 progeny of the remaining one-third were exclusively hygromycin-resistant and GUS-positive. The R_2 progeny of the hygromycin-sensitive, GUS-negative R_1 plants from plant 10c were all sensitive to the drug and negative for GUS activity.

Table 4. Estimation of copy numbers of transgenes in the transformants produced by EHA101(pIG121Hm), and segregation patterns for hygromycin resistance and expression of GUS in the progeny

	Copy number in R_0 generation		Hygromycin resistance			GUS expression			
			Number of plants in R_1 generation		Number of loci	Number of plants in R_1 generation			Number of loci
Transformant[a]	HPT	GUS	R	S		+	C	−	
1a	1	1	58	22	1	15		5	1
1b	NT	NT	42	18	1	14		6	1
2	4	6	48	22	1	13		7	1
3a	2	2	28	2	2	18		2	1–2
3b	NT	NT	29	1	2–3	19		1	2
4a	4	5	27	3	1–2	18		2	1–2
4b	NT	NT	39	1	2–3	20		0	2–3
5	2	2	30	0	2–3	19		1	2–3
6a	2	1	46	4	2	14		6	1
6b	NT	NT	37	3	2	15		5	1
6c	NT	NT	38	2	2–3	12		8	1
7a	2	2	42	18	1	17		3	1–2
7b	2	2	32	8	1	13		7	1
8a	1	1	76	24	1	15		5	1
8b	1	1	36	14	1	15		5	1
9	2	2	23	7	1	15		5	1
10a	2	1	36	14	1	17		3	1–2
10b	2	1	32	8	1	16		4	1
10c	2	1	72	28	1	14		6	1
11	6	6	89	41	1	2	14	4	?
12a	1	1	40	10	1	15		5	1
12b	1	1	70	20	1	13		7	1
13	2	2	47	13	1	16		4	1
14	4	4	30	10	1	15		5	1
15	1	1	54	26	1	15		5	1
16a	2	2	42	18	1	15		5	1
16b	2	2	31	9	1	15		5	1
16c	NT	NT	21	9	1	14		6	1
16d	NT	NT	31	9	1	15		5	1
17a	2	1	45	5	2	17		3	1–2
17b	NT	NT	40	0	2–3	15		5	1
18a	3	3	29	21	?	7	6	7	?
18b	3	3	48	12	1	16		4	1
19a	3	2	26	14	1	17		3	1–2
19b	3	2	36	14	1	17		3	1–2
19c	3	2	31	9	1	17		3	1–2
19d	NT	NT	27	13	1	15		5	1
19e	NT	NT	8	32	?	0	13	7	?
20	2	2	47	13	1	16		4	1

[a]Transformants with designations that differ only in the letters of the alphabet were regenerated from a single colony of cells.
R, resistant; S, susceptible; C, chimera; NT, not tested.

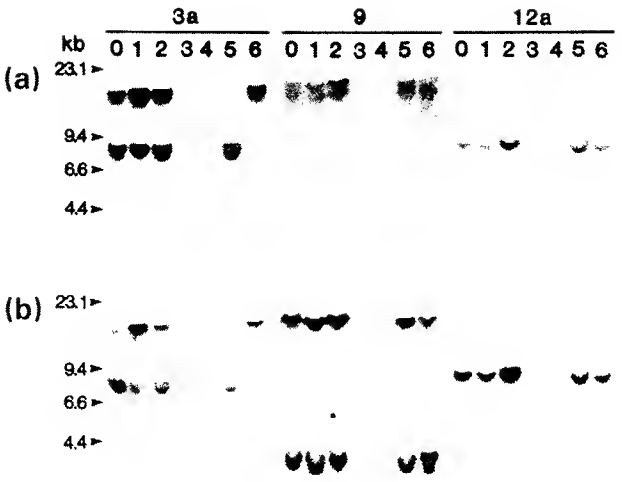

Figure 4. Southern analysis of the R_1 progeny of transformants 3a, 9 and 12a.
DNA from R_0 transformants (0), hygromycin-resistant and GUS-positive R_1 progeny (1, 2, 5, and 6), and hygromycin-sensitive and GUS-negative R_1 progeny (3 and 4) was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *hpt* (a) or the *gus* (b) probe.

Analysis of the boundaries of the T-DNA

The junction regions between rice genomic DNA and T-DNA were amplified from selective transformants and nucleotide sequences were determined. The sequences of three right boundaries and five left boundaries from rice that had been transformed with strain EHA101(pIG121Hm) are shown in Figure 5 together with the sequence of the T-DNA borders of pTiT37 (Yadav *et al.*, 1982; Zambryski *et al.*, 1982), which is the source of the T-DNA borders in pIG121Hm. One of the right boundaries corresponded precisely to the site found in tobacco transformants (Yadav *et al.*, 1982; Zambryski *et al.*, 1982). The two other right junctions had two more bases that might have originated from T-DNA. The five left boundaries were in the 25 bp repeat region like the left boundaries found in tobacco transformants.

Discussion

The advantages of *Agrobacterium*-mediated gene transfer over other methods that can be used for the transformation of higher plants include the high efficiency of transformation, the transfer of pieces of DNA with defined ends, the transfer of relatively large segments of DNA, and the absence of a requirement for protoplast-culture techniques. Therefore, this type of gene transfer is normally the method of choice when more than one method is available. The ability of *Agrobacterium* to transform monocotyledons has been the subject of a serious debate for some time, since these plants are not natural hosts of this bacterium. The controversy now appears to be resolved, at least for rice, since the present study and that of Chan *et al.* (1993) have clearly demonstrated the stable integration of foreign DNA in rice chromosomes in a process mediated by *A. tumefaciens*, as well as the Mendelian transmission of the DNA to the progeny. We obtained hundreds of independent transformants of three cultivars of japonica rice, and more than 20 plants were characterized in detail.

Various factors affected the efficiency of transformation. Optimization of the conditions of tissue culture and of the condition of co-cultivation was of a critical importance. The composition of the medium was especially important for the koshihikari cultivar, which generally

Right boundary	T-DNA	right 25-bp border repeat	outside T-DNA
pTiT37	--TAAACTATCAGTGTTTGACAGGATATATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTA--		
tobacco	--TAAACTATCAGTGTTTnnnnnnnnnnnnnnnnnnnn--		
rice 11	--TAAACTATCAGTGTTTGAgctcctcctcctctgtcgcc--		
rice 12a	--TAAACTATCAGTGTTTGAtctggtctttttcctttgtt--		
rice 30	--TAAACTATCAGTGTTTaaagaggaataaaaaaatta--		

Left boundary	T-DNA	left 25-bp border repeat	outside T-DNA
pTiT37	--TAAGCGTCAATTTGTTTACACCACAATATATCCTGCCACCAGCCAGCCAACAGCTCCCC--		
rice 2	--TAAGCGTCAATTTGTTTACACCACAATATAgcgaaatattaagtgcgccg--		
rice 10a	--TAAGCGTCAATTTGTTTACACCAgtgagacgggcaacagctgat--		
rice 11*	--TAAGCGTCAATTTGTTTACccccatccacgttccgattt--		
rice 11*	--TAAGCGTCAATTTGTTTACACCACAATATAgcagagctaattatggagta--		
rice 12a	--TAAGCGTCAATTTGTTTACACCACAATATATCCaacgtcaaagggcgatttat--		

Figure 5. Sequence analysis of T-DNA/plant DNA junctions.
Sequences of the junctions found in selective rice transformants are shown below the sequences of the T-DNA borders of pTiT37. Sequences presumably originated from rice genomic DNA are shown in lower case letters, and bases that could originate from either T-DNA or rice genomic DNA are shown in italic. A consensus sequence for right junctions in tobacco transformants is shown between the pTiT37 and rice sequences. 'n' stands for any nucleotide.
*Rice plant 11 had multiple copies of T-DNA and the sequences of two left boundaries were determined.

Table 5. Media for culture of rice tissues

Medium	Composition
AAM	AA salts and amino acids (Toriyama and Hinata, 1985), MS vitamins (Murashige and Skoog 1962), 500 mg l^{-1} casamino acids, 68.5 g l^{-1} sucrose, 36 g l^{-1} glucose, 100 μM acetosyringone, pH 5.2.
N6F	N6 major salts, N6 minor salts, and N6 vitamins (Chu 1978), sucrose 20 g l^{-1}, 2 g l^{-1} Gelrite (Merck), pH 5.8.
N6F-H	N6F medium plus 70 mg l^{-1} hygromycin.
N6S3	Half-strength N6 major salts, N6 minor salts, N6 vitamins, AA amino acids, 1 g l^{-1} casamino acid, 20 g l^{-1} sucrose, 0.2 mg l^{-1} naphthaleneacetic acid, 1 mg l^{-1} kinetin, 3 g l^{-1} Gelrite, pH 5.8.
N6S3-AS	N6S3 medium plus 10 g l^{-1} glucose and 100 μM acetosyringone, pH 5.2.
N6S3-CH	N6S3 medium plus 250 mg l^{-1} cefotaxime and 50 mg l^{-1} hygromycin.
N6-7-CH	N6 major salts, N6 minor salts, N6 vitamins, 2 g l^{-1} casamino acid, 20 g l^{-1} sucrose, 30 g l^{-1} sorbitol, 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} 6-benzyladenine, 100 mg l^{-1} hygromycin, 250 mg l^{-1} cefotaxime, 2 g l^{-1} Gelrite, pH 5.8.
N6-7K-CH	Half-strength N6 major salts, N6 minor salts, N6 vitamins, 2 g l^{-1} casamino acids, 200 ml l^{-1} potato extract[a], 20 g l^{-1} sucrose, 30 g l^{-1} sorbitol, 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} 6-benzyladenine, 100 mg l^{-1} hygromycin, 250 mg l^{-1} cefotaxime, 2 g l^{-1} Gelrite, pH 5.8.
2N6	N6 major salts, N6 minor salts, N6 vitamins, 1 g l^{-1} casamino acids, 30 g l^{-1} sucrose, 2 mg l^{-1} 2,4-D, 2 g l^{-1} Gelrite, pH 5.8.
2N6L	2N6 medium without Gelrite.
2N6-AS	2N6 medium plus 10 g l^{-1} glucose and 100 μM acetosyringone, pH 5.2.
2N6-CH	2N6 medium plus 250 mg l^{-1} cefotaxime and 50 mg l^{-1} hygromycin.
2N6K-CH	Half-strength N6 major salts, N6 minor salts, N6 vitamins, 1 g l^{-1} casamino acids, 200 ml l^{-1} potato extract, 30 g l^{-1} sucrose, 2 mg l^{-1} 2,4-D, 250 mg l^{-1} cefotaxime, 50 mg l^{-1} hygromycin, 2 g l^{-1} Gelrite, pH 5.8.

[a]Potato extract was prepared as follows: 200 g of potato tubers were peeled, cut into pieces of 1–2 cm^3, mixed with 1 l of water and autoclaved at 120°C for 10 min. Then the supernatant was added to media as indicated.

shows poor responses in tissue culture, during the preliminary stage of this study. Since many high-quality cultivars of japonica rice are related to Koshihikari, the methods described here should be very useful in rice breeding. Important variables related to the conditions of co-cultivation include addition of acetosyringone to the media and a temperature between 22°C and 28°C; transformation was not successful when acetosyringone was omitted or co-cultivation was carried out at a temperature higher or lower than that range (data not shown). Although Chan *et al.* (1993) indicated that the addition of medium from a suspension culture of potato cells to the co-cultivation medium was essential, our methods did not require such additions.

The choice of tissues as starting material was one of the most important factors. Various tissues were screened for their responses to co-cultivation with *A. tumefaciens*. GUS expression in the tissues immediately after infection offered a good indication of preferable tissues. Pretreatment of tissues, for example, by wounding or enzymatic digestion of cell walls, was not necessary. Such pretreatments were found to be essential in other studies (Chan *et al.*, 1993; Moony *et al.*, 1991; Raineri *et al.*, 1990). The *intron-gus* (Ohta *et al.*, 1990) was a convenient marker gene since this gene is strongly expressed in rice cells but not in *A. tumefaciens* cells that attach to the tissues. Early expression of GUS was observed in tissues that included shoot apices and immature embryos, tissues that were successfully transformed in previous studies (Chan *et al.*, 1993; Gould *et al.*, 1991; Raineri *et al.*, 1990). However, only a few transformants were obtained from immature embryos and none were obtained from shoot apices. The results of this study clearly indicate that callus cultures initiated from scutella are excellent materials for transformation of rice by *Agrobacterium*.

The patterns generated by Southern hybridization differed among our transformants, strongly indicating that T-DNAs were randomly integrated in the rice genome. The DNA fragments that hybridized to the *hpt* and *gus* probes clearly did not originate from contaminating *A. tumefaciens* used in the transformation because the vectors in strains employed would have given bands of fragments of defined sizes, for example, a 15 kb band from pIG121Hm, in the analysis. Rearrangement of DNA, for example, deletion of part of the T-DNA, was occasionally observed, as is also the case in *Agrobacterium*-mediated gene transfer in dicotyledons (Deroles and Gardner, 1988; Komari, 1989, 1990a).

Genetic analysis of the R_1 and R_2 progeny also pro-

vided conclusive evidence of the incorporation of T-DNA into rice chromosomes. Hygromycin resistance and GUS expression were inherited by the R_1 and R_2 offspring in Mendelian fashions, and the data from Southern analysis of the R_1 generation supported the genetic data. The progeny of a limited number of transformants showed unusual patterns of segregation. Since these lines included plants that were chimeric for GUS expression, it appeared that the chimerism in the R_0 generation affected the segregation ratios. Chimerism in transformants produced by *A. tumefaciens* has also been reported in tobacco (Schmülling and Schell, 1993) but has not been a limiting factor in the application of the technique.

Sequence analysis of the junctions between T-DNA and plant DNA in the rice transformants revealed that the T-DNA boundaries in rice were essentially the same as those in dicotyledons (Yadav *et al.*, 1982; Zambryski *et al.*, 1982). This evidence provides further support for the hypothesis that T-DNAs are transferred from *Agrobacterium* to dicotyledonous and monocotyledonous plants by an identical molecular mechanism.

The performance of so-called 'super-virulent' strains of *A. tumefaciens* has been emphasized in previous reports (Chan *et al.*, 1993; Gould *et al.*, 1991; Raineri *et al.*, 1990), as well as in this study. Since strains that carried pTiBo542, a 'super-virulent' Ti plasmid, were reported to operate very efficiently in transformation (Jin *et al.*, 1987; Komari, 1989), two new types of system based on pTiBo542 have been developed. The first involves strain EHA101 (Hood *et al.*, 1986), which carries a 'dis-armed' version of pTiBo542, and this strain has been popular in trials aimed at transformation of monocotyledons. The second involves what is known as a 'super-binary' vector, in which a DNA fragment from the virulence region of pTiBo542 is introduced into a small, T-DNA-carrying plasmid (Komari, 1990b) used in a binary vector system (An *et al.*, 1988; Hoekema *et al.*, 1983). In this study, we tested combinations of two strains and two vectors. The strains were an 'ordinary' strain LBA4404 (Hoekema *et al.*, 1983), and a 'super-virulent' strain EHA101. The vectors were pIG121Hm, a derivative of the 'normal' binary vector pBIN19 (Bevan, 1984), and pTOK233, a derivative of a 'super-binary' vector pTOK162 (Komari, 1990b).

In transformation experiments, LBA4404(pTOK233) was slightly more effective than both LBA4404(pIG121Hm) and EHA101(pIG121Hm) with cv. Tshukinohikari and it was definitely the most effective with cv. Koshihikari. Contrary to our expectations, EHA101(pTOK233) was, for unknown reasons, not very effective for transformation of rice. These data suggest that an 'ordinary' vector/strain combination is similar to improved combinations in the transformation of cultivars that are 'easy' to grow in tissue culture, while the choice of vectors and strains is important for transformation of 'difficult' cultivars. Since LBA4404(pTOK233) transformed the two types of cultivar equally efficiently, strains such as LBA4404(pTOK233) will be very useful in genetic engineering of rice. We even have preliminary data to suggest that LBA4404(pTOK233) might also be able to transform cultivars of indica rice.

We have developed a method for consistent production of stable transformants of rice of three cultivars at frequencies between 10 and 30% using scutellum-derived calli inoculated with *A. tumefaciens*. It was easy to produce more than 20 independent transformants in a single series of experiments without complicated manipulations in tissue culture. The efficiency of the method seems similar to that of the transformation of dicotyledons with the bacterium. Furthermore, the majority of plants produced by this method were free of morphological aberrations, probably as a result of the fact that the cells were maintained in culture *in vitro* for a short time, about 4 months from the initiation of callus to the transfer of regenerants to soil, as compared with the protoplast-mediated methods, which require at least 6 months. Therefore, *Agrobacterium*-mediated gene transfer is now available as a straightforward and routine method for the genetic modification of rice.

Experimental procedures

Rice cultivars and culture media

Cultivars of japonica rice, namely, *Oryza sativa* L. Tsukinohikari, Asanohikari and Koshihikari, were used in transformation. Media used for tissue culture are listed in Table 5.

Shoot apices, roots and calli derived from roots

Mature seeds were dehusked, sterilized with 70% ethanol for 1 min and then with 1.5% (w/v) NaClO for 30 min. They were rinsed thoroughly with sterile water and allowed to germinate on N6F medium at 25°C in darkness for 3 days. Segments of 0.5 mm × 1 mm, consisting of shoot apices plus two or three leaves, and segments (5–10 mm in length) of roots were excised from the germinating seeds and used for transformation experiments. Calli were induced by culturing some of the root segments on 2N6 medium at 30°C in darkness for 3 weeks. Actively growing pieces of calli (about 1 mm in diameter) were used for transformation experiments.

Scutella, calli derived from scutella, and suspension cultures

Mature seeds, dehusked and sterilized as described above, were cultured on 2N6 medium at 30°C in darkness. After 5 days, scutella were excised from some of the seeds and used for transformation experiments. After 3 weeks, proliferated calli derived from scutella of the remaining seeds were subcultured on fresh 2N6 medium for 4 days. Actively growing pieces of calli

(1–2 mm in diameter) were used for transformation experiments. Pieces of calli derived from scutella were suspended in 2N6L medium and cultured on a rotary shaker (125 r.p.m.) at 25°C in darkness. The medium was changed every 7 days. Cells in the logarithmic phase of growth (4 days after the third or fourth subculture) were used for transformation experiments.

Immature embryos

Developing seeds were collected 10 days after anthesis, hulled, and sterilized with 70% ethanol and 1.5% NaClO. Then immature embryos (about 1 mm in length) were excised aseptically and used for transformation experiments.

Bacterial strains and plasmids

Agrobacterium tumefaciens strains LBA4404 (Hoekema *et al.*, 1983) and EHA101 (Hood *et al.*, 1986) have been described previously. Plasmids were introduced into these strains by electroporation (Sambrook *et al.*, 1989). *A. tumefaciens* was grown on AB medium (Chilton *et al.*, 1974) at 28°C.

A gene for GUS, which has an intron in the N-terminal region of the coding sequence and is connected to the 35S promoter of cauliflower mosaic virus, was constructed by Ohta *et al.*, (1990). This *intron-gus* reporter gene expresses GUS activity in plant cells but not in the cells of *A. tumefaciens* (Ohta *et al.*, 1990). pIG121Hm (Figure 1) is a binary vector that contains a kanamycin-resistance gene (*npt*), a hygromycin-resistance gene (*hpt*), and the *intron-gus* in the T-DNA region. pIG121Hm was introduced into LBA4404 and EHA101.

pIG221 (Ohta *et al.*, 1990) was digested with *Eco*RI, treated with T4 DNA polymerase, and circularized with a *Hin*dIII linker (5'-CAAGCTTG-3'). The resultant plasmid was digested with *Hin*dIII and the 3.1 kb fragment, which contained the *intron-gus*, was inserted into the *Hin*dIII site of pGL2, a derivative of pUC18 (Yanisch-Perron, *et al.*, 1985) that contained *hpt*, to generate pGL2-IG. pTOK162 (Komari, 1990b), a binary vector that contained *virB* and *virG* from Ti-plasmid pTiBo542, was introduced into LBA4404 and EHA101. pGL2-IG was subsequently introduced into LBA4404(pTOK162) and EHA101(pTOK162). The resultant strains contained a co-integrated form of pTOK162 and pGL2-IG generated by homologous recombination. The co-integrated form is referred to as pTOK233 (Figure 1). Thus, pTOK233 contained *npt*, *hpt* and *intron-gus* in the T-DNA region as did pIG121Hm.

Transformation

A. tumefaciens strains LBA4404(pIG121Hm), EHA101(pIG121Hm), LBA4404(pTOK233) and EHA101(pTOK233) were grown for 3 days on AB medium supplemented with 50 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin. The bacteria were collected with a small spoon, and suspended at a density of 3–5 × 10^9 cells ml^{-1} in AAM medium.

The rice tissues described above were immersed in the bacterial suspension for several minutes and then transferred without rinsing on to 2N6-AS medium (all tissues except for shoot apices) or N6S3-AS medium (shoot apices), and incubated at 25°C in darkness for 3 days. After the co-cultivation, the materials were rinsed thoroughly with 250 mg l^{-1} cefotaxime in sterile water and placed on 2N6-CH medium (all tissues from Asanohikari and Tsukinohikari apart from shoot apices), N6S3-CH (shoot apices), or 2N6K-CH (all tissues from Koshihikari), and cultured for 3 weeks. Colonies of cells that had proliferated on the first selection medium were excised and cultured on N6-7-CH medium (tissues from Asanohikari and Tsukinohikari) or N6-7K-CH (tissues from Koshihikari) for 10 days. Colonies of cells that had proliferated were plated on a regeneration medium, N6S3-CH, and incubated at 25°C under continuous illumination (about 2000 lux). Regenerated plants (R_0 generation) were eventually transferred to soil in pots and grown to maturity in a greenhouse.

Test of the progeny for resistance to hygromycin

Selfed seeds (R_1 and R_2 generations) of transformants were sown on N6F medium and, 2 or 3 days later, the seeds were transferred to N6F-H medium and cultured under continuous illumination at 25°C. Drug resistance was scored 7 days after the transfer; the shoots and roots of resistant seedlings grew normally on the selection medium.

Assay of GUS activity

Expression of GUS in rice cells was assayed essentially as described by Rueb *et al.* (1989) with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as substrate. Segments of rice tissues were incubated in phosphate buffer (50 mM $NaPO_4$, pH 6.8) that contained 1% Triton X-100 at 37°C for 1 h. The buffer was removed and fresh phosphate buffer containing 1.0 mM X-Gluc and 20% methanol was added to the segments. The reaction mixture was placed under a mild vacuum for 5 min, incubated overnight at 37°C, and then tissues were examined visually. Cells that expressed GUS were dark blue. Leaf segments were washed twice in 99% methanol for 2 h before the visual examination.

Isolation of DNA and Southern hybridization

DNA was extracted from leaf tissues by the procedure described by Komari *et al.* (1989). DNA (5–20 μg) was digested with *Hin*dIII and fractionated on a 0.65% agarose gel by electrophoresis at 1.5 V cm^{-1} for 15 h. Southern hybridization was carried out as described by Sambrook *et al.* (1989). The probe for *hpt* was the 1.1 kb *Bam*HI fragment from pGL2-IG and the *gus* probe was the 1.9 kb *Sal*I–*Sac*I fragment from pGL2-IG.

Sequencing of border regions of the inserted T-DNA

Junction regions of the introduced T-DNA and rice genomic DNA were analysed using cassette-ligation-mediated polymerase chain reactions (PCR), as described by Isegawa *et al.* (1992). The cassette and cassette primers were purchased from Takara (Kyoto, Japan).

DNA isolated from the transformants was digested with *Mbo*I and ligated to a *Sau*3AI cassette that consisted of the sequences 5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCAC TATAGGGA-3' and 3'-CATGTATAACAGCAATCTTGCGCATTA TGCTGAGTGATATCCCTCTAG-5'.

The reaction mixture (50 μl) for the first PCR consisted of an aliquot of the ligase reaction mixture (50 ng of template DNA), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dGTP, dATP, dTTP and dCTP, 1 unit of

Taq DNA polymerase and 10 pmol each of cassette primer C1 (5'-GTACATATTGTCGTTAGAACGCG-3') and primer LS1 (5'-TCAGTACATTAAAAACGTCCGCA-3', for the left border) or primer RS1 (5'-GATCAGATTGTCGTTTCCCG-3', for the right border). Thermal cycling for 1 min at 94°C, 1 min at 60°C and 1 min at 72°C was performed for 30 cycles.

The second PCR was carried out using 1 μl of the reaction mixture from the first PCR as template, cassette primer C2 (5'-TAATACGACTCACTATAGGGAGA-3'), and primer LS2 (5'-ACCATGGATCCGCAATGTGTTATTAAGTT-3', for the left border) or primer RS2 (5'-TAGTCAGATCTGTCGTTTCCCGC CT-3', for the right border). The composition of the reaction mixture and the details of thermal cycling were the same as those for the first PCR apart from the template and primers. The products of PCR were cloned into a cloning vector, pCRII (Invitrogen, San Diego, CA), and sequenced by a standard procedure (Sambrook *et al.* 1989)

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References

An, G., Evert, P.R., Mitra, A. and Ha S.B. (1988) Binary vectors. In *Plant Molecular Biology Manual* A3 (Gelvin, S.B. and Schilperoort R.A., eds). Dordrecht: Kluwer Academic Press, pp. 1–19.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711–8721.

Bytebier, B., Deboeck, F., DeGrave, H., van Montagu, M. and Hernalsteens, J.-P. (1987) T-DNA organization in tumor cultures and transgenic plants of monocotyledon *Asparagus officiallis*. *Proc. Natl Acad. Sci. USA*, **84**, 5345–5349.

Chan, M.-T., Lee, T.-M. and Chang, H.-H. (1992) Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiol.* **33**, 577–583.

Chan, M.-T., Chang, H.-H., Ho, S.-L., Tong, W.-F. and Yu, S.-M. (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. *Plant Mol. Biol.* **22**, 491–506.

Chilton, M.-D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P. and Nester, E.W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl Acad. Sci. USA*, **71**, 3672–3676.

Chu, C.-C. (1978) The N6 medium and its applications to anther culture of cereal crops. In *Proc. Symp. Plant Tissue Culture*. Peking: Science Press, pp. 43–50.

Deroles, S.C. and Gardner, R.C. (1988) Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. *Plant Mol. Biol.* **11**, 365–377.

Gould, J., Devery, M., Hasegawa, O., Ulian, E.C., Peterson, G. and Smith, R.H. (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol.* **95**, 426–434.

Grimsley, N.H., Ramos, C., Hein, T. and Hohn, B. (1988) Meristematic tissues of maize plants are most susceptible to Agroinfection with maize streak virus. *Bio/Techniques*, **6**, 185–189.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature*, **303**, 179–180.

Hood, E.E., Helmer, G.L., Fraley, R.T. and Chilton, M.-D. (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in *a* region of pTiBo542 outside of T-DNA. *J. Bacteriol.* **168**, 1291–1301.

Isegawa, Y., Sheng, J., Sokawa, Y., Yamanishi, K., Nakagomi, O. and Ueda, S. (1992) Selective amplification of cDNA sequence from total RNA by cassette-ligation-mediated polymerase chain reaction (PCR): application to sequencing 6.5-kb genome segment of hantavirus strain B-1. *Mol. Cell. Probes*, **6**, 467–475.

Jin, S., Komari, T., Gordon, M.P. and Nester, E.W. (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. *J. Bacteriol.* **169**, 4417–4425.

Komari, T. (1989) Transformation of callus cultures of nine plant species mediated by *Agrobacterium*. *Plant Sci.* **60**, 223–229.

Komari, T., Saito, Y., Nakakido, F. and Kumashiro, T. (1989) Efficient selection of somatic hybrids in *Nicotiana tabacum* L. using a combination of drug-resistance markers introduced by transformation. *Theor. Appl. Genet.* **77**, 547–552.

Komari, T. (1990a) Genetic characterization of a double-flowered tobacco plant obtained in a transformation experiment. *Theor. Appl. Genet.* **80**, 167–171.

Komari, T. (1990b) Transformation of cultured cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542. *Plant Cell Rep.* **9**, 303–306.

Li, X.-Q., Liu, C.-N., Ritchie, S.W., Peng, J.-Y., Gelvin, S.B. and Hodges, T.K. (1992) Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. *Plant Mol. Biol.* **20**, 1037–1048.

Mooney, P.A., Goodwin, P.B., Dennis, E.S. and Llewellyn, D.J. (1991) *Agrobacterium tumefaciens*-gene transfer into wheat tissues. *Plant Cell Tissue Organ Cult.* **25**, 209–218.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Ohta, S., Mita, S., Hattori, T. and Nakamura, K. (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol.* **31**, 805–813.

Potrykus, I. (1990) Gene transfer to cereals: an assessment. *Bio/Technology*, **8**, 535–542.

Raineri, D. M., Bottino, P., Gordon, M. P. and Nester, E. W. (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.) *Bio/Technology*, **8**, 33–38.

Rueb, S. and Hensgens, L.A.M. (1989) Improved histochemical staining for β-o-glucuronidase activity in monocotyledonous plants. *Rice Genet. Newsl.* **6**, 168–169.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schläppi, M. and Hohn, B. (1992) Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. *Plant Cell*, **4**, 7–16.

Schmülling, T. and Schell, J. (1993) Transgenic tobacco plants regenerated from leaf disks can be periclinal chimeras. *Plant Mol. Biol.* **21**, 705–708.

Shen, W.-H., Escudero, J., Schläppi, M., Ramos, C., Hohn, B. and Koukolikova-Nicola, Z. (1993) T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize tissues. *Proc. Natl Acad. Sci. USA*, **90**, 1488–1492.

Toriyama, K. and Hinata, K. (1985) Cell suspension and protoplast culture in rice. *Plant Sci.* **41**, 179–183.

Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M. and Chilton, M.D. (1982) Short direct repeats flank the T-DNA on a nopaline ti plasmid. *Proc. Natl Acad. Sci. USA*, **79**, 6322–6326.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

Zambryski, P., Depicker, A., Kruger, K. and Goodman, H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J. Mol. Appl. Genet.* 1, 361–370.

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Transgenic Central American, West African and Asian Elite Rice Varieties Resulting from Particle Bombardment of Foreign DNA into Mature Seed-derived Explants Utilizing Three Different Bombardment Devices

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We developed a novel system based on particle bombardment for the transfer of foreign genes into eight important rice varieties grown in Central America, West Africa and Asia. Novel features of this improved protocol, which we believe will replace alternative systems currently utilizing immature embryos, embryogenic cell lines or suspension cultures include: (*a*) bombardment of target tissue 5 d after seed germination; (*b*) no requirement to select for embryogenic callus prior to bombardment; (c) high transformation frequencies (up to 14%); (*d*) ability to recover transgenic material at similar frequencies irrespective of bombardment devices used. Southern blot analysis and enzymatic assays in primary transformants and progeny confirmed the stable integration and expression of introduced transgenes in all engineered cultivars.

Key words: *Oryza sativa*, transgenic rice, particle bombardment, transformation.

INTRODUCTION

Rice is the most important staple food crop for most of the world's population, particularly in developing countries in Central and South America, West Africa, the Indian subcontinent, China and SE Asia. Indica cultivars are mostly cultivated in developing countries and are of significant economic importance. The development of efficient gene transfer protocols for delivering cloned genes into cultivated varieties of rice is an important component of any rice improvement programme. Progress in practical applications of plant molecular biology has been dependent on the development of efficient and practical methods to introduce foreign DNA into plant tissues, which subsequently could be regenerated to intact plants relatively easily. Introduction of foreign genes into plant cells can be achieved routinely by either direct DNA transfer or using biological vectors such as *Agrobacterium*. During the last few years, introduction of foreign DNA into rice cells, with subsequent recovery of transgenic plants and progeny expressing the introduced trait(s) stably over a number of generations, has been described by many laboratories worldwide.

Reports of the recovery of the first transgenic rice plants were published in 1988 by three groups, utilizing protoplasts and electroporation or PEG fusion (Toriyama *et al.*, 1988; Zhang and Wu, 1988; Zhang *et al.*, 1988). Datta *et al.* (1990) published the first report of the recovery of transgenic indica plants from the variety Chinsurah Boro II. Christou, Ford and Kofron (1991, 1992) developed a variety-independent method based on bombardment of immature embryos that led to the recovery of fertile transgenic plants from many varieties (both indicas and japonicas) at high frequencies. Hiei *et al.* (1994) recovered transgenic rice plants using *Agrobacterium*. The first successful rice field trial using a gene of agronomic importance was reported in 1996 (Oard *et al.*, 1996). Subsequently, many reports describing recovery of transgenic rice appeared in the literature. The range of transformed genotypes and transformation efficiencies has expanded and improved significantly since these original reports.

Recent advances in the *Agrobacterium* field (e.g. Hiei *et al.*, 1994; Aldemita and Hodges, 1996) indicate that we are now in a position to engineer rice using either direct DNA transfer or *Agrobacterium*-mediated transformation. A number of reports describing organization of transgenes (Kohli *et al.*, 1998), mechanism of DNA integration after the bombardment process (Kohli *et al.*, pers. comm.) and stability of expression in relation to transgene copy number (Hiei *et al.*, 1994; Qu *et al.*, 1996, Kohli *et al.*, pers. comm.) indicate that there are no significant differences in the molecular and biochemical make up of plants generated by either method. This is, however, still a contentious issue and it is apparent that much more careful experimentation needs to be carried out to settle issues including transgene stability over a number of generations, particularly in the field, and the influence of transgene copy number on expression.

We present here a very efficient rice transformation protocol which removes many of the remaining constraints to make the technology available to all those interested in engineering cultivated local rice varieties effectively.

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MATERIALS AND METHODS

Seeds from eight commercial cultivars, Orion (American japonica), CR-5272 (Costa Rican indica), A-92 (Mexican indica), IR-64 (Class I indica, IRRI), ASD16 (S Indian indica), ITA212 (West African tropical japonica) and M9 and M7 (javanicas) were dehusked and surface-sterilized in 70% ethanol for 3 min, then in a 4·5% sodium hypochlorite solution for 35 min, and rinsed five times in sterile distilled water. Fifteen seeds were placed in a Petri dish containing 40 ml of culture medium for callus induction: MS basal medium supplemented with 2·5 mg l^{-1} 2,4-D, 3% (w/v) sucrose, and solidified with 0·2% (w/v) Gelrite. The pH of the medium had been previously adjusted to 5·8 before autoclaving at 120 °C for 20 min. Seeds were incubated in the dark at 27 °C. Mature embryos were separated from the endosperm 5 d after incubation. Twenty-five embryos were placed on the centre of a Petri dish containing the same induction medium immediately prior to bombardment, with the scutellum orientated in such a way to be in the direct path of the accelerated particles carrying the DNA. Plasmids pWRG1515 [containing both the *gusA* and the hygromycin-resistance genes, under the control of the 35S Cauliflower Mosaic Virus (CaMV) promoter (Christou *et al.*, 1992)] and pWRG2426 [(containing the *bar* resistance gene in addition to the *hpt* and the *gusA* genes, all driven by their own 35s CaMV promoters; Cooley, Ford and Christou, 1995)] were described previously. Plasmids were purified by CsCl gradient centrifugation.

Three different particle bombardment devices were compared. These are designated I, II and III and correspond to the conventional BioRad particle bombardment device, helium-driven PDS-1000/He (I), the electric discharge, ACCELL device (II; Agracetus, Inc., Madison, Wisconsin, USA) and a new simple and portable particle inflow gun (III), respectively. For device I, DNA coating and bombardment procedures were essentially those described by Cabrera-Ponce, Vegas-Garcia and Herrera-Estrella (1995) with some modifications: 50 ml M-17 tungsten particles (15 mg ml^{-1}), 10 ml DNA (1 μg ml^{-1}), 50 ml 2·5 M $CaCl_2$ and 20 ml 0·1 M spermidine were mixed in sequential order, then briefly sonicated. The mixture was centrifuged at 10000 rpm for 10 sec. The supernatant was removed and the pellet was resuspended in 250 ml 100% ethanol. The pellet recovered after a second centrifugation (10000 rpm, 10 sec) was resuspended in 60 ml 100% ethanol. After a short sonication, aliquots of 10 ml were delivered onto the centre of each macrocarrier. The tissue was bombarded twice at 1200 psi (4 h interval between bombardments). For device II, the procedure described in Vain *et al.* (1998*a*) was followed. Important differences to the procedure described earlier include use of gold instead of tungsten, bombardment at 16 kV, incorporation of a pre- as well as a post-bombardment osmoticum treatment of the tissue and utilizing a Helium atmosphere in the bombardment chamber to minimize aerodynamic drag of the particles. For device III, bombardments were carried out at atmospheric pressure, using gold, and incorporating a pre- and a post-bombardment treatment, as above. Gold particles were coated with DNA as described by Christou *et al.* (1991). The suspension was sonicated briefly and then injected into an appropriate length of teflon tubing (3 mm id). The gold was allowed to settle under gravity at room temperature for about 5 min. The alcohol was then drained carefully and the tubing was inserted into a glass tube and rotated at high speed (300 rpm) to distribute the gold evenly on the inner circumference. It was extremely important to dry the tubes completely before proceeding to the next step since any residual moisture would result in uneven particle delivery during shooting, or in extreme cases would prevent dislodgement of the particles carrying DNA by the He gas after firing. After drying, the tube was cut into 1·25 cm segments (charges) and stored dry at −20 °C until needed. The loaded charges were inserted into the particle inflow gun (Corbett Scientific, Sydney, Australia) and the gun was then connected to a helium cylinder through high pressure metal tubing and charged to the appropriate pressure. We found a pressure of 450 psi was optimal for effective particle and DNA delivery into target tissue. The accelerating force was provided by a rapid and instantaneous discharge of the trigger, which releases the appropriate helium pressure. The gas pressure was subsequently directed through the charges holding the gold-DNA precipitate and it is this force that dislodges and accelerates the particles to impact the target tissue, at atmospheric pressure. GUS histochemical assays were performed according to Jefferson, Kavanagh and Bevan (1987). PAT assays were carried out following the procedure of De Block *et al.* (1987). Two days after bombardment, embryos were transferred to selection medium MS callus induction medium supplemented with hygromycin (30–50 mg l^{-1}) or PPT (3 mg l^{-1}) and incubated in the dark at 27 °C for 3–4 weeks. At this stage, fast growing callus appeared and this was transferred to fresh medium under selection (secondary selection). After approximately 6–8 weeks, resistant callus was transferred to regeneration medium (MS basal medium supplemented with 2 mg l^{-1} BAP, 0·5 mg l^{-1} NAA, and the appropriate selective agent at the same levels as before. For selection involving PPT, some cultivars required removal of selection during the regeneration phase to accelerate growth. Selection pressure in such cases was re-applied at the rooting stage.

Genomic DNA was extracted from callus and leaf tissues of rice plants using procedures described earlier (Rogers and Bendich, 1994). For Southern blot analysis, 5 μg of genomic DNA from each sample was digested overnight at 37 °C with appropriate restriction endonucleases (Fig. 2) and separated on 1% agarose gels. Gels were transferred to nylon membranes (Hybond-N^+, Amersham) using a 2X SSC buffer. The hygromycin probe used was the 2·3 kb *Bam* HI fragment and the *gusA* probe was the 2·6 kb *Xho* I and *Stu* I fragment from pWRG 1515. The entire coding sequence of the *bar* gene was used as a probe. ^{32}P labelling, hybridization and washing conditions were as described by Cabrera-Ponce *et al.* (1995).

Autoradiography was carried out by using Kodak X-OMAT-AR film for 7 d at −70 °C. Segregation analysis for select R1 lineages was carried out at the DNA level using PCR and Southern blot analyses and also by evaluating resistance to hygromycin/PPT at the germination stage or GUS expression in leaf tissue of R1 seedlings.

RESULTS

Mature seeds germinated on MS basal medium containing 2·5 mg l^{-1} 2,4-D, 3% (w/v) sucrose, and solidified with 0·2% (w/v) Gelrite produced nodular structures derived from the general area of the scutellum (Fig. 1A). Morphology of the explant at this stage was very similar for all eight genotypes tested. Explants were separated from the endosperm 5 d after plating (Fig. 1B) and bombarded. A high frequency of GUS expression was observed in the scutellum region of bombarded explants (Fig. 1C). Bombarded mature embryos were subcultured every 2–3 weeks

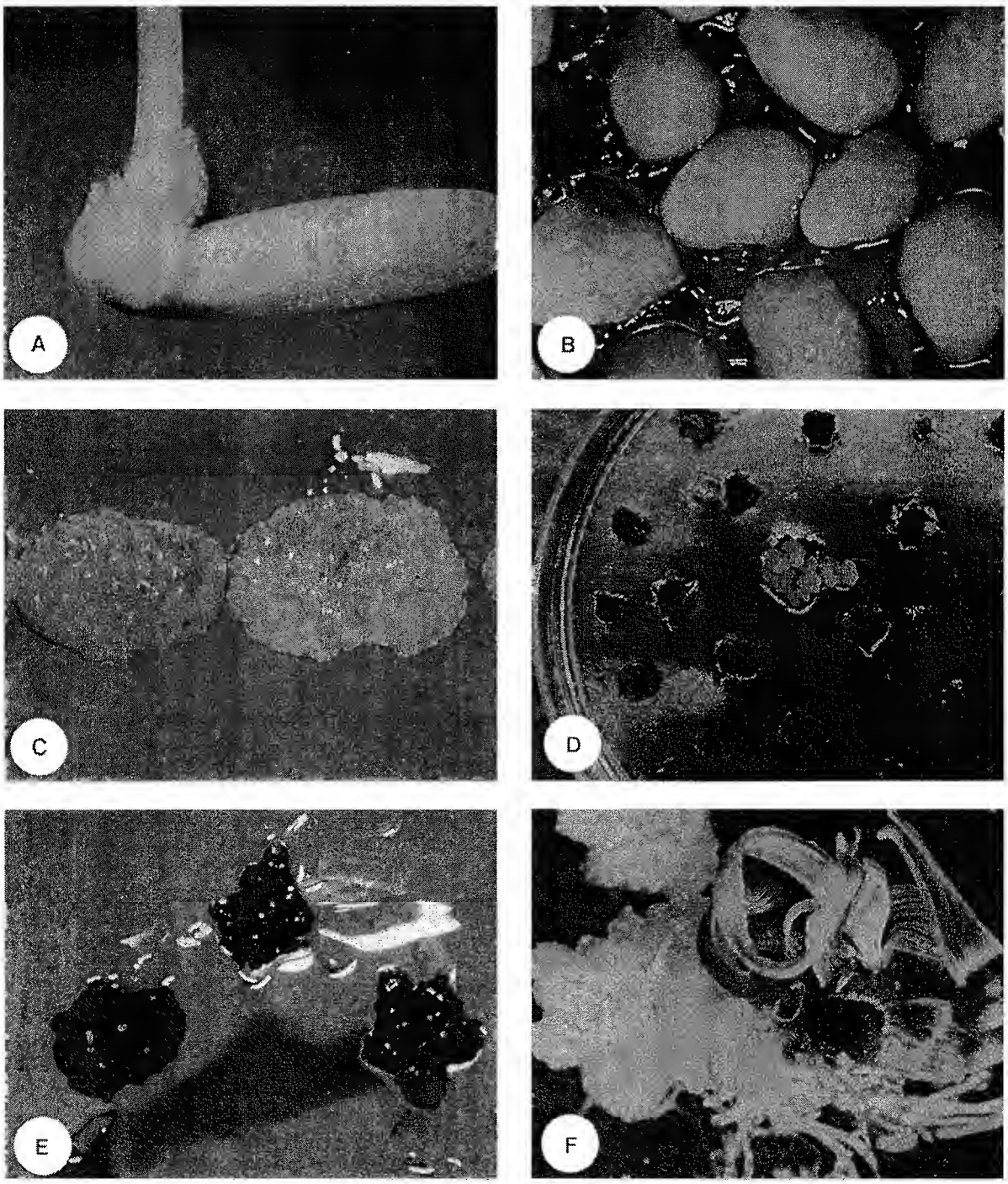

FIG. 1. Rice mature seed transformation system. A, Scutellum tissue 5 d after germinating seeds on induction medium; B, seed-derived embryos immediately prior to bombardment; C, transient GUS expression 48 h after bombardment; D, hygromycin resistant clone growing on selection medium 4 weeks after bombardment; E, GUS histochemical analysis of hygromycin-resistant clones; F, regenerating plants from resistant clone under selection.

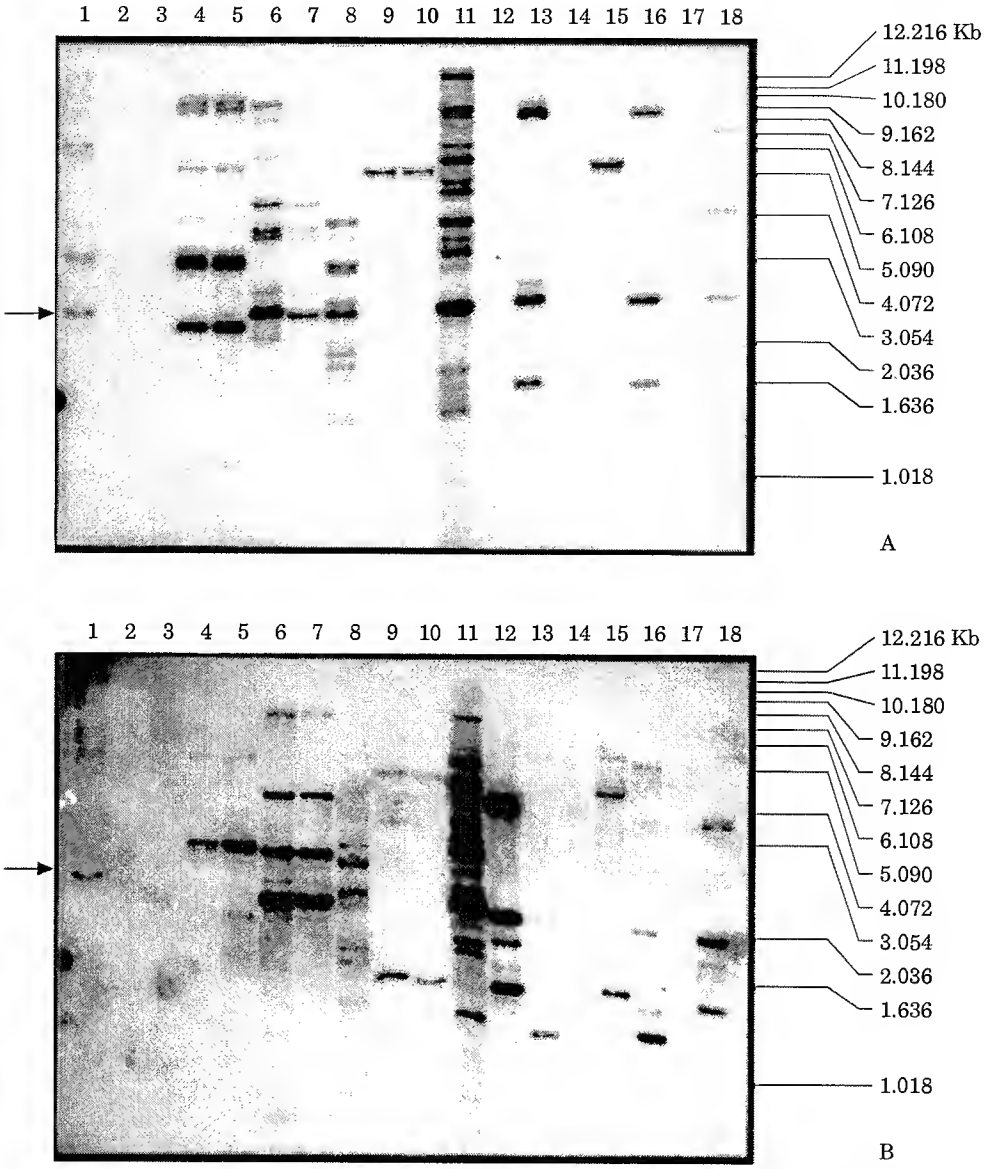

FIG. 2. Southern blot analysis of transgenic rice callus and plants probed for the *hpt* gene (A) and the *gusA* gene (B). The position of molecular weight markers is indicated on the right. Arrows indicate the 2·3 kb expected fragment for the *hpt* gene (promoter, coding sequence and terminator) following digest with *Xba*I and the 2·6 kb expected fragment for the *gusA* gene (coding sequence only) following digestion with *Hin*dIII. Lane 1, Positive control (digested plasmid); lanes 2 and 3, DNA from wild-type plants; lanes 4 and 5, IR-64 callus and plant derived from the same clone; lanes 6 and 7, CR-5272 callus and plant; lane 8, callus from Orion; lanes 9 and 10, callus and plant derived from Orion line; lanes 11, 12 and 15, plants from independent A-92 clones; lanes 13 and 16, callus and plant from CR-5272 clone; lane 18, independent CR-5272 clone (plant).

to fresh selection medium until resistant callus developed. Such callus could be identified easily after 6 weeks of culture (Fig. 1 D). Transformation efficiency using this system was found to range from 1·7 to 14% of bombarded embryos developing at least one hygromycin- or PPT-resistant callus line, which could subsequently be regenerated to transgenic plants. When such callus was stained for GUS activity, immediately prior to transfer onto regeneration medium, a blue colour developed rapidly on approx. 75% of the assayed callus pieces, as shown in Fig. 1 E. The remaining non-staining tissue was found to contain the gene when analysed molecularly as described in the Materials and Methods. After 6–8 weeks of selection, resistant callus was transferred to regeneration medium. This resulted in the recovery of plants as shown in Fig. 1 F.

Ten hygromycin- and 10 PPT-resistant rice clones and plants derived from them, and also selected progenym, were analysed in more detail for the presence of the *hpt*, *bar* and *gusA* genes. Figure 2 illustrates molecular analysis of transgenic callus and plants derived from them for *hpt* and *gusA* genes and Figure 3 shows a population of transgenic plants containing the *bar* gene.

Progeny analysis using PCR, Southern blots and resistance to selective agents/GUS expression confirmed

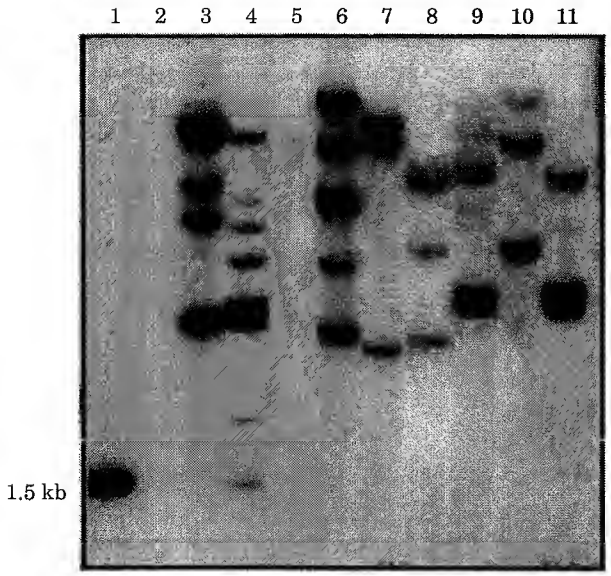

FIG. 3. Southern blot analysis of genomic DNA of transgenic plants containing the *bar* resistance gene. Lane 1, Positive control (1·5 kb fragment containing 35S promoter, *bar* gene and terminator digested from the plasmid); lane 2, wild type plant; lanes 3–11, transgenic plants.

Mendelian inheritance of introduced traits in progeny, in accordance with previous reports (Christou *et al.*, 1992; Cooley *et al.*, 1995). Table 1 lists transformation frequencies for the eight cultivars investigated in this study and Table 2 gives an overall impression of genetic analysis of selected lines from each genotype confirming Mendelian transmission of integrated transgenes to R_1 progeny.

DISCUSSION

In early experiments, immature rice embryos were isolated from glasshouse-grown plants and subjected to particle bombardment (Christou *et al.*, 1991). Bombarded tissue was plated on regeneration medium supplemented with appropriate selective agents (the antibiotic hygromycin B or the herbicide Basta® or its analogues). Appearance of callus expressing the introduced genes could be seen 5–7 d following bombardment. Continuous selection of the proliferating tissue on hygromycin-containing media resulted in the appearance of transformed embryogenic callus. In a number of experiments, it was observed that up to 50% of bombarded explants were capable of developing transformed embryogenic callus which could subsequently generate transformed embryos and other transgenic organized tissues, such as shoots and roots in addition to transformed plants. Stable transformation in R_0 plants and their progeny was confirmed by extensive molecular, biochemical and genetic analyses. When progeny from transgenic rice plants carrying the *bar* gene were sprayed with the herbicide bialaphos they were shown to express total resistance to the herbicide at levels of 2000 ppm.

Results from early experiments demonstrated conclusively that engineering of rice through transformation of organized tissue was possible and transformation frequencies were very high. In subsequent experiments improvements in the procedure permitted recovery of plants from additional cultivars at even higher frequencies (Christou *et al.*, 1992).

Sivamani *et al.* (1996) reported a gene transfer procedure for the model indica rice variety TN1, using bombardment of embryogenic callus. They developed a procedure which allowed generation of highly homogeneous populations of embryogenic callus by selectively propagating a small number of regeneration-proficient callus pieces derived from seed. It was possible to produce thousands of embryogenic callus pieces from approx. 50 seeds within 10 weeks. Efficiency of transformation was defined in terms of the number of hygromycin-resistant R_0 plants regenerated from 100 callus pieces bombarded under standard conditions. An efficiency of 3% could be obtained, on average, over a number of experiments.

Despite the significant advances in rice transformation over the last few years, further improvements in the system are possible and desirable, particularly now that the emphasis in rice biotechnology is shifting rapidly to applied aspects involving engineering of elite cultivated varieties, with genes conferring agronomic properties, particularly in rice-growing countries in Central America, West Africa and Asia. For the technology to be utilized routinely in conventional rice improvement programmes, it is important that it is simple, facile and efficient in terms of recovering large numbers of transgenic plants from any given cultivar with relatively little effort. Important advances include incorporation of visible markers such as GFP (Vain *et al.*, 1998*b*) to increase efficiency of selection, and the incorporation of a pre- and post-bombardment osmoticum treatment for the target tissue (Vain *et al.*, 1998*a*).

In this report, we describe significant modifications in the rice transformation procedure, using eight important cultivated rice varieties. These improvements make the process significantly more practical compared to previously published protocols and also confirm that rice transformation through bombardment is not dependent on the acceleration device used. The procedure circumvents most of the shortcomings of alternative methods using bombardment or *Agrobacterium* such as the use of immature embryos and the requirement for the development and maintenance of embryogenic suspension or callus cultures. In the present study, transformation frequencies for experiments in which the electric discharge gun was used ranged from 1·7–9·6%; for the BioRad 1000/He, transformation frequencies ranged from 3·1–14%, whereas the modified inflow gun resulted in transformation frequencies in the range of 4–9·6%. We have thus demonstrated that three alternative acceleration devices resulted in recovery of transgenic plants at comparable frequencies. This is important as our results validate the use of a simple, portable and inexpensive acceleration device which can be used by all those interested in rice engineering. In this procedure, 25 rice seeds are germinated on induction medium for 5 d. This results in the development of the scutellum region of the embryo, which is subsequently excised and subjected to bombardment using standard procedures. The tissue is then subcultured onto callus induction medium containing hygromycin, or PPT. Actively growing callus is subcultured on the same medium at 2–3

TABLE 1. *Transformed genotypes and frequencies*

Genotype	No. of bombarded explants	No. of independent hygromycin or PPT resistant cell lines	Transformation frequencies (%)	Particle gun used
Orion	124	8	6·5	PDS-1000/He
IR64	163	5	3·1	PDS-1000/He
A-92	92	13	14·0	PDS-1000/He
CR-5272	175	7	4·0	PDS-1000/He
ITA212	125	8	6·4	Accell
ASD16	120	2	1·7	Accell
M7	230	22	9·6	Accell
M9	100	4	4·0	Particle inflow gun
M7	114	11	9·6	Particle inflow gun

TABLE 2. *Segregation of transgene expression in R_1 progeny of 26 independent primary transformants*

Plant line	GUS	BAR
Orion-1	Not expressing	3:1
Orion-2	3:1	3:1
Orion-3	3:1	3:1
Orion-4	Not expressing	1:1
IR64-1	Not expressing	3:1
IR64-2	1:1	1:1
IR64-3	3:1	3:1
A-92-1	3:1	3:1
A-92-2	3:1	3:1
A-92-3	Not expressing	3:1
A-92-4	3:1	3:1
CR-5272-1	Not expressing	3:1
CR-5272-2	3:1	3:1
CR-5272-3	1:1	1:1
CR-5272-4	3:1	3:1
ASD16-1	Not expressing	3:1
ASD16-2	3:1	3:1
M9-1	Not expressing	3:1
M9-2	Not expressing	3:1
M9-3	3:1	3:1
M9-4	3:1	3:1
M7-1	1:1	1:1
M7-2	3:1	3:1
M7-3	3:1	3:1
M7-4	Not expressing	1:1
M7-5	1:1	1:1

Results are statistically different.

week intervals depending on the growth rate of the callus. As expected, different rice cultivars proliferate at different rates. When resistant tissue is established, it is then transferred to regeneration medium in the presence of the selective agent. In some cases, when using PPT for selection, it may be advantageous to remove selective pressure at this stage, to encourage rapid regeneration. Regenerating shoots can then be rooted in the presence of selective agent and transferred to soil following standard protocols. The transgenic nature of these plants is confirmed using molecular and biochemical analysis. Southern blot analyses of R_0 plants and their progeny demonstrated stable integration of exogenous genes into the rice genome (Figs 2 and 3). Plants exhibiting GUS activity and/or antibiotic or herbicide resistance, upon restriction endonuclease digestion of genomic DNA, were shown to contain fragments corresponding to the intact coding region of the enzymes (Figs 2 and 3). Digestion with other restriction endonucleases, which do not cut within the *gusA*, *bar* or *hpt* genes, allowed us to estimate copy number/integration sites for the transgenes.

A significant advantage in the protocol we describe in this report is the use of mature seed-derived explants for bombardment in combination with a number of different acceleration devices. This eliminates the requirement for the use of immature embryos, callus or suspension cultures in transformation experiments. By substituting mature seed-derived explants for immature embryos in the transformation protocol, the need to establish flowering plants all year round in order to have access to immature embryos is removed. Examples of problems range from uneven flowering, inconsistent availability of immature embryos, significant glasshouse costs, etc.

In summary, we believe the following components make the above procedure attractive for rice transformation: (*a*) utilization of mature seed as explant; (*b*) no requirement to select for embryogenic callus prior to bombardment; (*c*) no need to establish embryogenic suspension cultures; (*c*) high transformation frequencies in a genotype-independent manner (up to 14%); and (*d*) ability to recover transgenic material at similar frequencies irrespective of bombardment devices used.

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LITERATURE CITED

Aldemita RA, Hodges TK. 1996. *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. *Planta* **199**: 612–617.

Cabrera-Ponce JL, Vegas-Garcia A, Herrera-Estrella L. 1995. Herbicide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. *Plant Cell Reports* **15** 1–7.

Christou P, Ford T, Kofron M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology* **9**: 957–962.

Christou P, Ford TL, Kofron M. 1992. The development of a variety-independent gene-transfer method for rice. *Trends in Biotechnology* **10**: 239–246.

Cooley J, Ford T, Christou P. 1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. *Theoretical and Applied Genetics* **90**: 97–104.

Datta SK, Peterhans A, Datta K, Potrykus I. 1990. Genetically engineered fertile indica-rice recovered from protoplasts. *Bio/Technology* **8**: 736–740.

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Rao Movva N, Thompson C, Van Montagu M, Leemans J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO Journal* **6**: 2513–2518.

Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6**: 271–282.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. *GUS* fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**: 3901–3907.

Kohli A, Leech M, Vain P, Lauri DA, Christou P. 1998. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. *Proceedings of the National Academy of Sciences USA* **95**: 7203–7208.

Oard JH, Linscombe SD, Braverman MP, Jodari F, Bollich PK, Blouin DC, Leech M, Kholi A, Vain P, Christou P. 1996. Development, field evaluation and agronomic performance of transgenic herbicide resistant rice. *Molecular Breeding* **2**: 359–368.

Qu R, De Kochko A, Zhang L, Marmey P, Li L, Tian W, Zhang S, Fauquet CM, Beachy RN. 1996. Analysis of a large number of independent transgenic rice plants produced by the biolistic method. *In Vitro Cell and Developmental Biology—Plant* **32**: 233–240.

Rogers SO, Bendich AJ. 1994. Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin SB, Schilperoot RA, eds. *Plant molecular biology manual, 2nd edn* D1. The Netherlands: Kluwer Academic publishers, 1–8.

Sivamani E, Shen P, Opalka N, Beachy RN, Fauquet CM. 1996. Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. *Plant Cell Reports* **15**: 322–327.

Toriyama K, Arimoto Y, Uchimiya H, Hinata K. 1988. Transgenic rice plants after direct gene transfer into protoplasts. *Bio/Technology* **6**: 1072–1074.

Vain P, Worland B, Clarke MC, Richard G, Beavis M, Liu H, Kohli A, Leech M, Snape J, Christou P, Atkinson H. 1998*a*. Expression of an engineered cysteine proteinase inhibitor (Oryza cystatin-I 86) in transgenic rice plants. *Theoretical and Applied Genetics* **96**: 266–271.

Vain P, Worland B, Kohli A, Leech M, Snape J, Christou P. 1998*b*. The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. *Theoretical and Applied Genetics* **96**: 164–169.

Zhang W, Wu R. 1988. Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. *Theoretical and Applied Genetics* **76**: 835–840.

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR. 1988. Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. *Plant Cell Reports* **7**: 379–384.

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Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*

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A rapid *Agrobacterium tumefaciens*-mediated transformation system for wheat was developed using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. The explants were inoculated with a disarmed *A. tumefaciens* strain C58 (ABI) harboring the binary vector pMON18365 containing the β-glucuronidase gene with an intron, and a selectable marker, the neomycin phosphotransferase II gene. Various factors were found to influence the transfer-DNA delivery efficiency, such as explant tissue and surfactants present in the inoculation medium. The inoculated immature embryos or embryogenic calli were selected on G418-containing media. Transgenic plants were regenerated from all three types of explants. The total time required from inoculation to the establishment of plants in soil was 2.5 to 3 months. So far, more than 100 transgenic events have been produced. Almost all transformants were morphologically normal. Stable integration, expression, and inheritance of the transgenes were confirmed by molecular and genetic analysis. One to five copies of the transgene were integrated into the wheat genome without rearrangement. Approximately 35% of the transgenic plants received a single copy of the transgenes based on Southern analysis of 26 events. Transgenes in T_1 progeny segregated in a Mendelian fashion in most of the transgenic plants.

In the early 1980s, the era of plant transformation was initiated when *Agrobacterium tumefaciens*-mediated gene delivery was reported for the production of transgenic plants (De Block et al., 1984; Horsch et al., 1984, 1985). Initial successes were limited to the Solanaceae, tobacco in particular. This dramatically changed throughout the 1980s and into the 1990s, and it is now possible to transform a wide range of plants, including many agronomically important crops such as soybean, cotton, peanut, and pea (Hinchee et al., 1988; Umbeck et al., 1989; Schroeder et al., 1993; Cheng et al., 1996). Although *A. tumefaciens*-mediated transformation has significant advantages over naked DNA delivery, such as introduction of a few copies of genes into the plant genome, high co-expression of introduced genes, and easy manipulation in vitro, the *A. tumefaciens*-mediated transformation method for gene transfer has been limited to dicotyledonous plants (Songstad et al., 1995).

Several reports presented early attempts to transform the Gramineae with *A. tumefaciens*, including *A. tumefaciens*-mediated infection of plants with viral genomes (Grimsley et al., 1988; Raineri et al., 1990; Gould et al., 1991; Mooney et al., 1991; Chan et al., 1992, 1993; Schläppi and Hohn, 1992; Shen et al., 1993). Chan et al. (1993) first reported the production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain and proved the transformation by molecular and genetic analysis. Recently, significant progress was made in *A. tumefaciens*-mediated transformation of rice and maize: a large number of transgenic plants were regenerated and characterized (Hiei et al., 1994; Ishida et al., 1996). Convincing and unambiguous data on transgene expression, gene segregation in the progeny, and DNA analysis were presented in these papers.

There have been limited studies on *A. tumefaciens*-mediated transformation of wheat (*Triticum aestivum* L.). Hess et al. (1990) pipetted *A. tumefaciens* into the spikelets of wheat, and several kanamycin-resistant grain progeny were obtained. However, the protocol was not reproducible and the Southern hybridization was not convincing in this study. Deng et al. (1990) infected the base of the leaf sheath and spike stem of wheat plants with several wild-type *A. tumefaciens* strains and opine-synthesizing tumors formed from these tissues. Mooney et al. (1991) infected the immature embryos of wheat with *A. tumefaciens* and a few kanamycin-resistant colonies were generated.

Here we present an *A. tumefaciens*-mediated transformation method for wheat using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. We produced a large number of transgenic plants and demonstrated stable integration, expression, and inheritance of transgenes in wheat plants.

MATERIALS AND METHODS

Stock Plants and Explant Tissues

A spring wheat, *Triticum aestivum* cv Bobwhite, was used throughout this study. Stock plants were grown in an environmentally controlled growth chamber with a 16-h photoperiod at 800 μmol m^{-2} s^{-1} provided by high-intensity discharge lights (Sylvania, GTE Products Corp., Manchester, NH). The day/night temperatures were 18/16°C. Immature caryopses were collected from the plants 14 d after anthesis. Immature embryos were dissected aseptically and cultured on a semisolid or liquid CM4 medium (Zhou et al., 1995) with 100 mg L^{-1} ascorbic acid (CM4C).

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Abbreviations: MS, Murashige-Skoog; T-DNA, transfer-DNA.

The MS salts (Murashige and Skoog, 1962) in the CM4C medium were adjusted to full strength (the original amounts) or one-tenth-strength (Fry et al., 1987). The immature embryos were cultured on these media for 3 to 4 h (freshly isolated) or 1 to 6 d (precultured). Embryogenic calli were prepared by culturing the immature embryos on CM4C medium for 10 to 25 d. The callus pieces derived from immature embryos were inoculated with *A. tumefaciens* without being broken down (intact), or only the embryogenic callus sectors were selected and separated into small pieces (approximately 2 mm).

A. tumefaciens Strain, Plasmid, and Culture

Disarmed *A. tumefaciens* C58 (ABI) harboring binary vector pMON18365 (Fig. 1) was used for all the experiments. pMON18365 contains the GUS (*uid*A) gene with an intron and the NPT II gene as a selectable marker within the T-DNA region. Each gene was under the control of an enhanced 35S (E35S) promoter. Cultures of *A. tumefaciens* were initiated from glycerol stocks and grown overnight at 25 to 26°C with shaking (150 rpm) in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 50 mg L^{-1} kanamycin, streptomycin, spectinomycin, and 25 mg L^{-1} chloramphenicol with 200 μM acetosyringone, to mid-log phase (OD_{660} = 1–1.5). The *A. tumefaciens* cells were collected by centrifugation and resuspended in liquid inoculation medium (CM4C with one-tenth-strength MS salts and supplemented with 10 g L^{-1} Glc and 200 μM acetosyringone). The *A. tumefaciens* cell density was adjusted to give an A_{660} of 1 to 2 for inoculation.

Inoculation and Co-Cultivation

The immature embryos and embryogenic calli maintained on the CM4C medium as described above were transferred into an *A. tumefaciens* cell suspension in Petri dishes. A surfactant (0.01–0.075% [v/v] Silwet, Monsanto, St. Louis, MO) or pluronic F68 (0.01–0.2% [w/v] Sigma) was added to the inoculation medium in some experiments. The inoculation was conducted at 23 to 25°C for 3 h in the dark. After inoculation the *A. tumefaciens* cells were removed by vacuum or with a transfer pipette, and the explants were placed on semisolid or on a filter paper wetted with liquid CM4C with one-tenth-strength or full-strength MS salts and supplemented with 10 g L^{-1} Glc and 200 μM acetosyringone. The co-cultivation was performed at 24 to 26°C in the dark for 2 or 3 d.

Selection and Regeneration of Transgenic Plants

After co-culture the infected immature embryos and calli were cultured on the solid CM4C medium with 250 mg L^{-1} carbenicillin for 2 to 5 d without selection. *A. tumefaciens*-infected explants were then transferred to CM4C medium supplemented with 25 mg L^{-1} G418 and 250 mg L^{-1} carbenicillin for callus induction. Two weeks later, the explants were transferred to the first regeneration medium, MMS0.2C (consisting of MS salts and vitamins, 1.95 g L^{-1} Mes, 0.2 mg L^{-1} 2,4-D, 100 mg L^{-1} ascorbic acid, and 40 g L^{-1} maltose, solidified by 2 g L^{-1} gelrite) supplemented with 25 mg L^{-1} G418 and 250 mg L^{-1} carbenicillin.

At transfer to the regeneration medium, each piece of callus derived from one immature embryo or one piece of inoculated callus was divided into several small pieces (approximately 2 mm). In another 2 weeks, young shoots and viable callus tissues were transferred to the second regeneration medium, MMS0C, which contains the same components as MMS.2C with all antibiotics except 2,4-D included. When the shoots developed into about 3-cm or longer plantlets, they were transferred to larger culture vessels containing the second regeneration medium for further growth and selection. Leaf samples were taken from some of the plantlets for the GUS histochemical assay at this stage. Plants that were highly G418 resistant or GUS positive were transferred to soil. All of the plants derived from the same embryo or piece of callus were considered to be clones of a given event.

GUS Histochemical Assay

GUS activity was assayed histochemically in a 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution using the buffer described by Jefferson (1987) except that 20% methanol was added to eliminate the endogenous GUS activity.

Functional Assay of NPT II Genes

Paromomycin Spray

T_1 seeds harvested from each T_0 plant were planted in 2-inch pots grown under the same conditions as the stock plants as described above. When plants reached the 3-leaf stage, they were spayed with 2% (w/v) paromomycin (Sigma) plus 0.2% (v/v) Tween 20. One week later the plants were evaluated for paromomycin damage. The plants with a functional NPT II gene showed no bleached spots, whereas the plants without a functional NPT II gene exhibited bleached spots throughout. Paromomycin was used in this assay and the leaf-bleach assay as described in the following section because it is a similar aminoglycoside antibiotic to G418, and is more effective and less expensive for these assays.

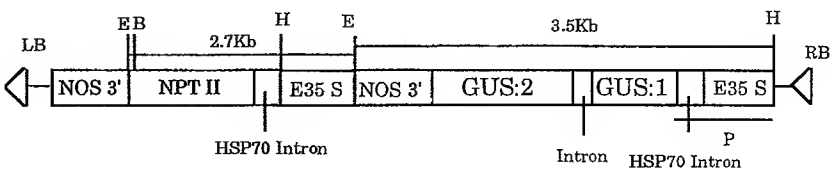

Figure 1. T-DNA regions of pMON 18365. RB, Right border; LB, left border; E35S, enhanced 35S promoter; HSP 70 intron, maize heat-shock protein 70 gene intron; NOS-3', 3' signal of nopaline synthase; NPT II, neomycin phosphotransferase II; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; P, probe.

Leaf-Bleach Assay

After the T_0 plants were established in soil, leaf samples (5–7 mm long) were taken from the youngest fully expanded leaves and placed in a 24-well culture plate (Costar, Cambridge, MA). Each well was filled with 0.5 mL of a water solution composed of 300 mg L^{-1} paromomycin and 100 mg L^{-1} fungicide (Benlate, DuPont) or 100 mg L^{-1} fungicide only. Three leaf samples taken from the same leaf of each plant were placed in two wells containing paromomycin and fungicide and one well containing fungicide only, respectively. Leaf samples from the nontransformed cv Bobwhite plants at a similar developmental stage were used as a negative control. The samples were vacuum-infiltrated in a desiccator using an in-house vacuum system for 5 min and then the plates were sealed with Parafilm before being placed under light for 3 d. The leaf samples that were highly resistant to paromomycin remained green in most of the area except around the edges (<1 mm wide), indicating that the NPT II gene was functional. The leaf samples from the plants without the gene or with a nonfunctional gene were bleached completely by paromomycin (as were the negative controls) (Fig. 2D) or had only small patches of green areas.

DNA Analysis

Genomic DNA was isolated from leaf tissue of T_0 plants and T_1 progeny following the method of Roger and Bendich (1985). An equal amount of *Eco*RI-digested genomic DNA (15 μg per lane) was separated on an agarose gel, blotted onto a membrane, and probed with a ^{32}P-labeled fragment containing the enhanced 35S promoter and the 5′ intron of the heat-shock protein 70 gene from maize following the manufacturer's protocol for the GeneScreen Plus membrane (DuPont).

Progeny Analysis

The segregation of the GUS and NPT II genes in the progeny of T_1 or reciprocal crosses was determined by one of the following methods: (a) paromomycin spray on the T_1 seedlings and GUS histochemical assay on leaf tissue, as described above; (b) leaf-bleach assay on the T_1 seedlings at the two-leaf stage; and (c) GUS histochemical assay on the immature (17 d after anthesis or older) and mature seeds harvested from the T_0 plants. The immature seeds were sterilized in 10% (v/v) bleach (containing 5.25% sodium hypochlorite) for 15 min followed by three rinses with sterile water. The mature seeds were soaked in the water for several hours and then sterilized in 20% bleach for 40 min. Finally, the seeds were washed in sterile water three times for 30 min each. Each seed was longitudinally cut into two uneven parts. The embryo from the large part was isolated and cultured on the MMS0C medium for germination. The seedlings were eventually transferred to soil. The small part and the large part without the embryo were used for the GUS histochemical assay. The T_1 seeds with or without the functional GUS gene could be determined based on the GUS activity in the embryo and endosperm tissues (Fig. 2H). The plants in soil were also assayed by leaf-bleach assay for the NPT II activity and GUS histochemical assay at different stages. The data were then analyzed by the χ^2 test to determine the number of the functional GUS or NPT II gene loci.

RESULTS

Factors Influencing the Efficiency of T-DNA Delivery

Various factors influencing the efficiency of T-DNA delivery were evaluated in the preliminary experiments. These factors include different explant types, *A. tumefaciens* cell density for inoculation, inoculation and co-culture time period, co-culture medium, surfactants in the inoculation medium, and induction agents in the inoculation and co-culture media. Leaf tissue from young seedlings, immature inflorescences, freshly isolated immature embryos, or precultured immature embryos, embryogenic callus derived from immature embryos, and cells in suspension cultures derived from wheat cv Mustang were inoculated and co-cultured with *A. tumefaciens* ABI:pMON18365.

GUS expression was detected in all of the tissues after either 2 or 3 d of co-culture and a 2-d delay of selection. Highly efficient T-DNA delivery was observed on both freshly isolated immature embryos and precultured immature embryos when surfactant (Silwet) was present in the inoculation medium (Fig. 2, A and B). The GUS spots were present across all of the scutellum surface of freshly isolated immature embryos, whereas most of the GUS spots were localized on the areas starting to form callus in the precultured immature embryos. Leaf sections, when vacuum infiltration was applied during inoculation, showed high GUS activity. The suspension cells exhibited the highest-efficiency T-DNA delivery even without the addition of surfactant in the inoculation medium. Therefore, the suspension cells were chosen as a model system to optimize the transformation parameters for wheat (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data).

A moderate number of GUS spots were observed on the embryogenic callus, whereas the spots were usually larger than in the immature embryos. Higher *A. tumefaciens* cell density and a longer time for inoculation and co-culture usually yielded more efficient T-DNA delivery on various tissues or cells, but more cell damage was observed. The salt strength in the inoculation medium was also found to influence the T-DNA delivery. For example, when one-tenth-strength MS salts were used for the inoculation and co-culture medium, transient GUS expression was significantly higher on the freshly isolated immature embryos than when the full-strength MS salts were used.

Another significant factor influencing T-DNA delivery was the presence of a surfactant in the inoculation medium. Two types of surfactants were evaluated based on T-DNA delivery efficiency with different wheat tissues. Both Silwet and pluronic F68 were found to have a significant positive effect on the transient GUS expression on different explants, especially on the immature embryos (Table I; Fig. 2A). Silwet at 0.01% started to enhance the transient GUS

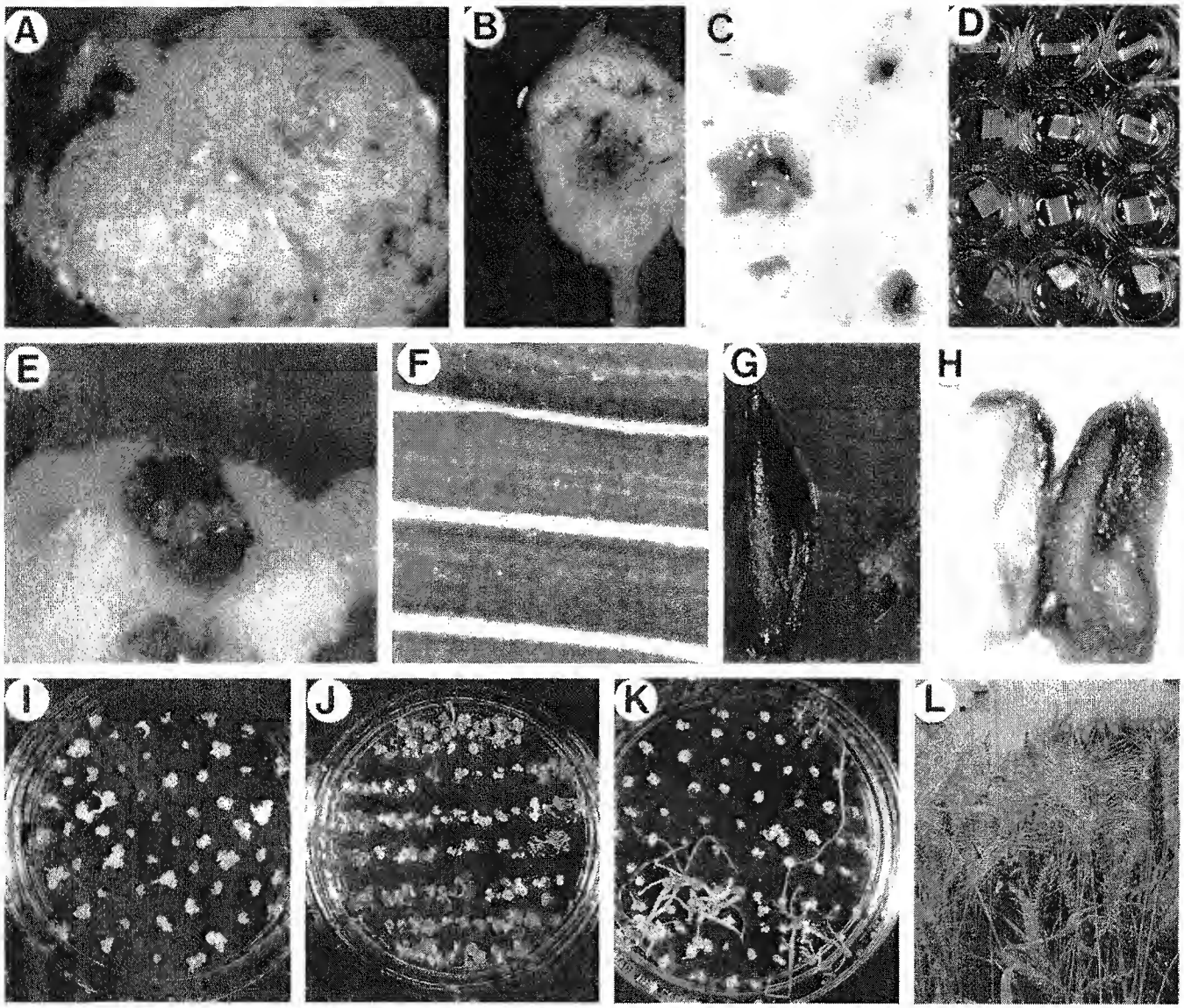

Figure 2. Transient GUS expression in *A. tumefaciens*-infected explants, stable GUS expression in various tissues from transgenic plants, and steps in the regeneration of transgenic plants. A, Transient GUS expression in a freshly isolated immature embryo 4 d after inoculation. B, Transient GUS expression in a precultured immature embryo 3 d after inoculation. C, Transient GUS expression in embryogenic calli 5 d after inoculation. D, Leaf-bleach assay. The wells of the first column (left) contained 100 mg L^{-1} fungicide-water solution and the remaining wells contained the 300 mg L^{-1} paromomycin and 100 mg L^{-1} fungicide-water solution. The first three rows of wells included leaf samples from three transgenic events with functional NPT II activity. The last column (bottom) was a leaf sample from a nontransgenic plant as a control. E, GUS expression in stably transformed, embryo-like tissue. *A. tumefaciens*-infected freshly isolated immature embryo was cultured on G418-containing CM4C medium for 3 weeks. F, GUS expression on young leaf tissue from a transgenic plant. G, GUS expression in a young ovary and glume tissues of a transgenic plant. H, Segregation of the GUS expression in T_1 seeds assayed at 20 d after anthesis from a GUS-positive T_0 plant. Some seeds showed GUS activity in both the pericarp (the maternal tissue) and the aleurone layer (right), and others had GUS activity only in the pericarp (left). I, Callus induction on G418-containing CM4C medium. Inoculated immature embryos were cultured on selective callus-induction medium CM4C for 2 weeks. J, Shoot regeneration from embryogenic calli after 2 weeks of culture on first-regeneration medium MMS2C containing G418. K, Plantlet regeneration after the embryogenic calli or shoots were cultured on second-regeneration medium MMS0C containing G418 for 2 weeks. L, Transgenic T_0 plants set seeds in a growth chamber.

expression on the scutellum side of the embryos. The concentration of Silwet at 0.05% gave the highest transient GUS expression, approximately 19-fold higher than the control. However, when the concentration of Silwet was greater than 0.05%, most of the immature embryos could not survive. Based on this result, 0.01 to 0.02% Silwet was used routinely in our stable transformation experiments. Pluronic F68 at 0.01 to 0.05% had the same effect as Silwet

Table I. *Effect of surfactant when present in the inoculation medium on transient GUS expression in freshly isolated immature embryos (IE)*

Concentration of Surfactant (Silwet)	IEs with GUS Spots	GUS Spots/IE
% (v/v)	% of total	
0.00	11/34 (34)	7.8
0.01	15/19 (79)	17
0.05	13/13 (100)	149
0.1	8/8 (100)	111
0.5	4/4 (100)	140

on the transient GUS expression on the immature embryo explants. Although both Silwet and pluronic F68 enhanced the efficiency of T-DNA delivery on the precultured immature embryos and embryogenic calli, they were not as significant as in the immature embryos. Silwet and pluronic F68 at 0.02% increased the transient GUS expression approximately 4-fold in the embryogenic calli compared with the control. An average of 30 blue spots was observed on each embryogenic callus (14 d old, intact) (Fig. 2C).

The presence of induction agents such as acetosyringone and Glc in the inoculation and co-culture media was crucial for efficient T-DNA delivery on some of the explants. For example, when acetosyringone and Glc were absent in the inoculation and co-culture media, the T-DNA delivery efficiency was significantly reduced in the freshly isolated immature embryos.

Regeneration of Transgenic Wheat Plants from Various Explants

A. tumefaciens-infected immature embryos and embryogenic calli were cultured on callus-induction medium CM4C with G418 for selection. Two weeks after callus induction, approximately 30 to 80% of the immature embryos formed embryogenic callus (Fig. 2I), whereas the inoculated embryogenic calli proliferated further on this medium. The GUS assay on some of the explants at this stage showed that the transformed, embryo-like tissue had developed from some of the inoculated explants (Fig. 2E). Developed calli were then broken down into small pieces, and transferred to the first regeneration medium for further selection. Multiple green shoots (most of them were not transformed) regenerated rapidly from the embryogenic calli (Fig. 2J). After 2 weeks of selection on the first regeneration medium, all of the viable shoots and callus tissues were transferred to the second regeneration medium, MMS0C with G418, for further selection (Fig. 2K). On this medium the most likely transformed shoots showed high resistance to G418, whereas most of the nontransformed shoots were not able to grow rapidly. Finally, highly resistant plantlets were transferred to larger culture vessels for further growth and selection. The transformed plants usually grew vigorously and formed strong root systems on the G418-containing MMSOC medium. The plants that survived the selection were moved to soil when they were approximately 10 to 15 cm in length.

Identification of Transgenic Plants and Transformation Efficiency

Most of the transgenic plants were identified by the GUS assay on the leaf tissues while the plantlets grew in the regeneration medium. After they were moved to soil, different tissues were collected at various stages for additional histochemical GUS assay. Leaf samples were also collected after the plants survived in soil for the leaf-bleach assay. Most of the plants had visible GUS activity in different tissues (Fig. 2, F–H), although the younger leaf tissue had higher activity than the older tissue, and young floral tissue had higher activity than the leaf tissue. However, a few of the plants that showed no visible GUS activity in leaf tissue had relatively high GUS activity in young floral tissues such as young ovary, stigma, glume, and lemma.

All of the plants showing GUS expression also had NPT II activity determined by the leaf-bleach assay except one that showed high-NPT II activity but no detectable GUS activity in any of the tissues (Table IV, event 21). The co-expression of GUS and NPT II genes in the plants produced via *A. tumefaciens*-mediated transformation was over 98% (49/50, Table IV) in our study. In contrast, the co-expression of the gene of interest (including the GUS gene) and the NPT II gene in the plants generated using the biolistic method, with either co-bombardment or 2 genes in the same construct, was from 42 to 62% in our laboratory, based on the analysis of 343 events with 4 different genes of interest. Therefore, the co-expression of two genes in the transgenic plants was significantly higher with the *A. tumefaciens*-mediated transformation than with the biolistic method.

Transgenic plants produced from all three kinds of explants are summarized in Table II. The transformation efficiencies for the freshly isolated immature embryos, precultured immature embryos, and embryogenic calli were 1.12% ± 0.79 ($\bar{X}$% ± SE), 1.56% ± 1.19, and 1.55% ± 1.08, respectively, no significant difference in the transformation efficiency was shown among the three explant types, although it varied among experiments. Transgenic plants could be regenerated from all three explants; however, several experiments with all three explants actually failed to produce any transgenic plants. These experiments were not included in Table II. The freshly isolated immature embryos always showed efficient transient GUS expression when the surfactants were present in the inoculation medium, but they could not recover well after inoculation and co-cultivation.

Although many different media and co-culture conditions were attempted, the majority of the inoculated immature embryos failed to form embryogenic calli or formed very limited calli on the scutellum surface. Precultured embryos usually showed good transient GUS expression on areas starting to form callus, and also exhibited better culture response. Among these three explants, embryogenic callus cultured for more than 10 d in the callus-induction medium showed the best culture response. Usually, 100% of the explants continued to proliferate on the callus-induction medium with the selection agent present.

Table II. *Summary of transformation results using three kinds of explants*

Experiment	Explant[a]	Explants (A)	Transgenic Events (B)	Transformation Efficiency (B/A)
		no.		%
1	FIIE	160	1	0.6
2	FIIE	250	3	1.2
3	FIIE	700	1	0.14
4	FIIE	124	1	0.8
5	FIIE	140	2	1.4
6	FIIE	38	1	2.6
7	PCIE (1 d)	23	1	4.3
8	PCIE (3 d)	98	1	1.0
9	PCIE (3 d)	104	2	1.9
10	PCIE (3 d)	36	1	2.8
11	PCIE (5 d)	97	1	1.0
12	PCIE (6 d)	40	1	2.5
13	EC (10 d)	239	1	0.4
14	EC (10 d)	232	1	0.4
15	EC (14 d)	47	1	2.1
16	EC (15 d)	110	3	2.7
17	EC (17 d)	50	1	2.0
18	EC (21 d)	73	2	2.7
19	EC (25 d)	308	1	0.3

[a] FIIE, Freshly isolated immature embryo; PCIE, precultured immature embryo; EC, embryogenic callus. The number of days of the immature embryos cultured on callus induction medium (CM4C) prior to inoculation is given in parentheses.

Characterization of the T_0 Plants

Plants identified as transgenic were grown in a growth chamber and evaluated for morphology and fertility. More than 100 events were established in soil and examined (Fig. 2L). All of the plants were fertile or partially fertile. The majority (about 80%) of the transformed plants produced as many seeds as the seed-derived control plants.

DNA was extracted from leaf tissue of 26 T_0 plants derived from independent events, and digested with *Eco*RI and hybridized with a probe consisting of E35S and the maize HSP 70 intron sequence (Fig. 3). DNA from nontransformed plants used as a negative control showed no hybridization to the probe. Since the T-DNA of pMON18365 had two *Eco*RI sites, the 2.7-kb band represented the internal fragment with the NPT II gene cassette without nos 3′ (Fig. 1). All 26 transgenic events had the 2.7-kb band (Fig. 3; Table III).

Because the third *Eco*RI site must be derived from the wheat genome, the number of hybridizing bands around or greater than 3.5 kb reflected the number of copies of the integrated gene (GUS) in the plants unless repeats of multiple copies of the T-DNA had been integrated. All of the detected bands except the 2.7-kb band represented the fragments of more than 3.5 kb. The mobilities of the bands differed from plant to plant, indicating independent events and random integration. The copy number of the integrated gene (GUS) varied from 1 to 5 (Fig. 2; Table III). A single copy of the transgene (GUS) was carried by 35% of the plants (9/26), and 50% (13/26) contained two or three copies. Only 15% of the plants (4/26) carried four to five copies of the transgene.

Inheritance of Transgenes

The selfed and backcrossed progeny were evaluated for resistance to paromomycin and GUS expression in the T_1 seeds or T_1 plants. The segregation patterns of 50 events are shown in Table IV. Paromomycin-resistant and -sensitive seedlings or GUS-positive and -negative seeds or plants were clearly distinguishable by spraying the paromomycin on the seedlings or by histochemical GUS assay. A segregation ratio of 3:1 was observed for 22 out of 50 (44%) independent events, indicating a single functional GUS or NPT II gene locus. Twenty-two percent of the events (11/50) had two or more functional loci. Thirty-two percent (16/50) of the events had a non-Mendelian segregation pattern; that is the GUS or NPT II gene segregated at a 1:1 ratio, or the number of GUS-negative or paromomycin-sensitive plants was greater than the number of GUS-positive and paromomycin-resistant plants.

Six out of eight events (nos. 5, 15, 16, 17, 25, and 28) containing a single copy of the GUS gene based on Southern analysis showed a 3:1 segregation ratio of GUS-positive plants to GUS-negative T_1 plants. If more than one copy of

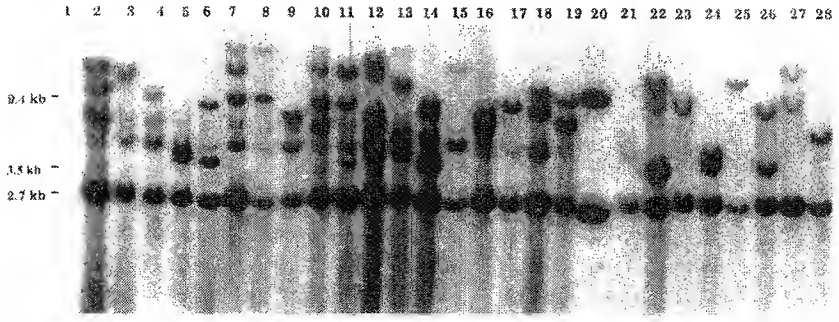

Figure 3. Southern analysis of T_0 transgenic events. DNA samples (15 μg) from 26 T_0 transgenic events (lanes 2–19 and 21–28; the lane number is the same as the event number) and one nontransformed wheat plant (lane 1) were digested with *Eco*RI, and the resulting fragments were resolved by electrophoresis and transferred to a membrane. The membrane was hybridized with a ^{32}P-labeled DNA probe corresponding to E35S promoter and the 5′ intron of maize HSP 70 gene. In lane 20, 5 ng of pMON18365 DNA digested with *Eco*RI was loaded as a positive control. The positions and lengths, in kilobars, of the molecular size markers are indicated.

Table III. *Copy number and functional loci of the GUS gene in transgenic events*

Events	Copy No. (GUS gene)	2.7-kb *Eco*RI	Functional Loci[a]
2	3	+	NA
3	2	+	?
4	3	+	1
5	1	+	1
6	2	+	1
7	4	+	2
8	2	+	1
9	2	+	1
10	3	+	?
11	4	+	?
12	5	+	2 or more
13	3	+	1
14	3	+	?
15	1	+	1
16	1	+	1
17	1	+	1
18	3	+	3
19	2	+	1
21	1	+	?
22	4–5	+	3 or more
23	1	+	?
24	1	+	NA
25	1	+	1
26	2	+	?
27	2	+	NA
28	1	+	1

[a] NA, Not analyzed; ?, the functional loci could not determined based on the segregation data because of the non-Mendelian segregation fashion in those events.

the gene was inserted in the plant genome, the estimated functional loci based on the segregation data were less than the copy number measured by Southern analysis in almost all of the cases (nos. 4, 6, 7, 8, 9, 13, and 19). The consistency of functional loci and the real copy number was observed in only one event (no. 18), which contained three functional loci and three copies of the gene.

The segregation ratios in the T_1 progeny from the reciprocal crosses of T_0 events 13, 28, 29, 30, and 60 are summarized in Table V. Events 13 and 28 had the 3:1 segregation ratio in the selfed progeny, whereas the progeny from the reciprocal crosses had a 1:1 segregation ratio. This result indicates that the transgenes were able to pass to the progeny through both male and female gametes.

The T_1 progeny from T_0 plants 18 and 28, which gave segregation patterns of 63:1 and 3:1 for GUS expression, respectively, were analyzed by Southern hybridization (Fig. 4). The T_0 plant 18 had three inserts, and two of the T_1 plants had exactly the same bands as their parent (lanes 3 and 4). Two other T_1 plants (lanes 5 and 6) had a band with the same size, and another band with a distinct size, indicating that the DNA coding the GUS gene segregated in the progeny. These results suggest at least two independent inserts in event 18. Because T_0 event 18 gave a segregation pattern of 63:1 for GUS expression, all three inserts should be independent. The T_0 event 28 had one insert (lane 7), and two of the T_1 plants (lanes 9 and 10) had the same pattern as their parent. One GUS-negative T_1 plant (lane 8) from T_0 event 18, which was selected as an example, did not show any hybridization signal.

Mendelian segregation for paromomycin resistance and GUS expression was also observed in the T_2 progeny plants 28 and 49 as an example. Two-thirds of the GUS-positive T_1

Table IV. *Segregation of the NPT II and GUS genes in the T_1 progeny*

Events	T_1 Plants Assayed by Paromomycin Spray			T_1 Plants Assayed for GUS Activity		
	Resistant (R)	Sensitive (S)	R/S	Positive (+)	Negative (−)	+/−
3	14	17	1:1	14	7	1:1
4	20	11	3:1	20	11	3:1
5	24	11	3:1	24	11	3:1
6	28	6	3:1	28	6	3:1
7	33	1	15:1	33	1	15:1
8	29	6	3:1	29	6	3:1
9	26	6	3:1	26	6	3:1
10	14	17	1:1	14	17	1:1
11	11	23	1:2	11	23	1:2
12	32	0	32:0	32	0	32:0
13	29	7	3:1	29	7	3:1
14	12	22	1:2	12	22	1:2
15	30	5	3:1	30	5	3:1
16	21	9	3:1	21	9	3:1
17	32	4	3:1	32	4	3:1
18	52	9	3:1	59	1	63:1
19				78	17	3:1
21	30	8	3:1	0	40	0:40
22				74	0	74:0
23				28	32	1:1
25				74	27	3:1
26				2	98	1:49
28	24	9	3:1	24	9	3:1
29	37	1	15:1	37	1	15:1
30	34	3	15:1	34	3	15:1
31	35	0	15:0	35	0	15:0
32	24	10	3:1	24	10	3:1
33	32	2	15:1	32	2	15:1
34	26	6	3:1	26	6	3:1
35	27	8	3:1	27	8	3:1
36	3	30	1:10	3	30	1:10
37	6	21	1:3	6	21	1:3
38	8	13	1:2	8	13	1:2
39	1	34	1:34	1	34	1:34
41	8	15	1:1	8	15	1:1
42	28	6	3:1	28	6	3:1
43	33	0	15:0	33	0	15:0
44	13	1	15:1	13	1	15:1
45	18	5	3:1	18	5	3:1
46	20	0	15:1	20	0	15:1
48	7	5	1:1	7	5	1:1
49	12	2	3:1	12	2	3:1
50	6	20	1:3	6	20	1:3
51	25	6	1:3	25	6	1:3
52	22	14	1:1	22	14	1:1
53	28	8	3:1	28	8	3:1
54	10	10	1:1	10	10	1:1
55	21	9	3:1	21	9	3:1
56				22	9	3:1
57	44	13	3:1	47	13	3:1

Table V. *Segregation of the NPTII gene in the progeny from the reciprocal crosses*

Crosses	Resistant Plants (R)	Sensitive Plants (S)	R:S
	no.		
No. 13 selfing	26	6	3:1
No. 13 X BW	8	3	1:1
BW X 13	20	13	1:1
No. 28 selfing	49	20	3:1
No. 28 X BW	11	15	1:1
BW X 28	5	8	1:1
No. 29 selfing	67	4	15:1
No. 29 X BW	9	2	3:1
BW X 29	10	4	3:1
No. 30 selfing	69	3	15:1
No. 30 X BW	22	9	3:1
BW X 30	16	4	3:1
No. 60 selfing	41	17	3:1
No. 60 X BW	16	12	1:1
BW selfing	0	76	NA

[a] BW, cv Bobwhite.

plants from both T_0 plants produced GUS-positive and GUS-negative T_2 at a ratio of 3:1. The T_2 progeny of the remaining one-third were exclusively GUS positive. The T_2 progeny of GUS-negative T_1 plants from both plants maintained the same expression pattern. These results suggested that the T_1 generation segregated into both homozygotic and heterozygotic plants, and that the transgenes were stably passed to their progeny in a Mendelian fashion.

DISCUSSION

We are reporting a rapid transformation method for wheat via *A. tumefaciens*. Our results showed strong evidence that the T-DNA was stably integrated into the wheat genome and transmitted to the progeny. Over 100 independent transformants have been regenerated, and one-half of them were characterized. This study and the studies on rice and maize transformation mediated by *A. tumefaciens* (Hiei et al., 1994; Ishida et al., 1996) have provided strong support that monocotyledons can be transformed as dicotyledons using *A. tumefaciens* by manipulating various factors such as explant tissues, inoculation, and co-culture conditions, as well as the *A. tumefaciens* strain and the combination of the *A. tumefaciens* strain and plasmid.

All of the studies of *A. tumefaciens*-mediated transformation of maize or rice used two strains, A281 or its derivative, EHA101 (Hood et al., 1986), and LBA4404 (Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Ishida et al., 1996; Rashid et al., 1996). The performance of the so-called "super-virulent" strain has been emphasized in some of the reports. Successful transformation of maize using *A. tumefaciens* was reported only when the "super-binary" vector was used. In the present study a nopaline *A. tumefaciens* strain C58 carrying the "ordinary" binary vector was used for infecting various explants, and this strain appeared to work efficiently. Stable transformants could be obtained from nonregenerable wheat suspension-cultured cells, and from regenerable immature embryos and embryogenic calli. Transgenic plants were successfully produced from all of the regenerable explants used.

Various factors influenced the T-DNA delivery and stable transformation efficiency. Inoculation and co-culture conditions can be varied so as to favor the plant cell survival. Different tissues or cells exhibited various abilities to survive after *A. tumefaciens* infection. For example, precultured immature embryos, embryogenic calli, and suspension cells, which were cultured for a period of time prior to inoculation, showed better survival than the freshly isolated immature embryos. Therefore, higher *A. tumefaciens* cell densities, higher concentrations of the surfactant, and longer amounts of time may be used for inoculating these explants. Acetosyringone and Glc can be added to the inoculation and co-culture media, particularly when using the freshly isolated immature embryos.

The T-DNA delivery efficiency was significantly decreased when acetosyringone was absent. The similar observation was also noticed in rice and maize transformation (Hiei et al., 1994; Ishida et al. 1996). However, in our study with wheat suspension-cultured cells, exogenous induction agents such as acetosyringone and Glc were not necessary for the stable transformation (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data). These results suggested that different tissues or cell types may have different competence for *A. tumefaciens* infection. Based on our results, the acetosyringone and Glc were recommended to be included in the inoculation and co-culture media for the stable transformation of the regenerable explants.

Surfactant present in the inoculation medium was one of the important factors noticed in this study. Two surfactants, Silwet and pluronic F68, proved to have a positive effect on the T-DNA delivery. The possible explanation for the effect the surfactants on enhancing T-DNA delivery might be the surface-tension-free cells favoring the *A. tumefaciens* attachment. We also tested other surfactants such

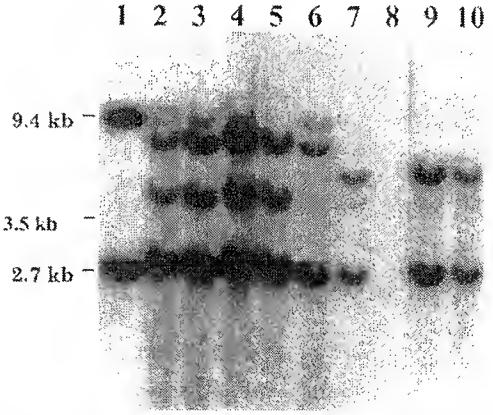

Figure 4. Southern analysis of T_1 progeny from T_0 plants 18 and 28. Southern blots were made as described in Figure 3. Lane 1, pMON18365 DNA as a positive control. Lane 2, DNA samples from T_0 plant 18. Lanes 3 through 6, DNA samples from T_1 progeny of T_0 plant 18. Lane 7, DNA sample from T_0 plant 28. Lane 8, DNA sample from GUS-negative T_1 plant of T_0 plant 28. Lanes 9 and 10, DNA samples from T_1 plants of T_0 plant 28. The positions and lengths, in kilobars, of the molecular size markers are indicated.

as Tween 20 and Triton X, which appeared to be too toxic to the wheat tissues even when only a small amount was added to the inoculation medium.

This transformation system was efficient and required only 2.5 to 3 months from inoculation to transfer of the plants to soil. The transformation efficiency was as high as 4%. Most of the published studies on wheat transformation by the biolistic method showed that it took a fairly long time for tissue culture and regeneration (from 12–28 weeks) (Vasil et al., 1993; Weeks et al., 1993; Becker et al., 1994; Nehra et al., 1994; Zhou et al., 1995; Ortiz, et al., 1996), and the transformation efficiency was from 0.1 to 5.7%. Altpeter et al. (1996) reported a protocol for accelerated production of transgenic wheat by particle bombardment in which 8 to 9 weeks were required to produce transgenic plants after the initiation of culture and the transformation efficiency was up to 2%. Using the same regeneration and selection protocol presented in this paper, up to 20% transformation efficiency can be achived in our laboratory through the biolistic approach. We think that once the inoculation and co-culture conditions are further optimized to obtain efficient T-DNA delivery with conditions favoring plant cell recovery, the transformation efficiency may be improved to as high as with the biolistic method.

Southern analysis showed different hybridization patterns among all of the tested T_0 transformants, indicating that T-DNAs were randomly integrated into the wheat genome. The T-DNA fragments that hybridized to the probe consisting of the E35S promoter and the maize HSP70 intron clearly did not derive from the vectors in the free *A. tumefaciens* cells that might exist in the plants regenerated from inoculated explants; otherwise, there would have been two bands, as in the control lane. Based on the samples tested, approximately 35% of the plants have single inserts, which was close to that observed in rice (32%) (Hiei et al., 1994), but significantly lower than that in maize (60–70%) (Ishida et al., 1996). The differences could be due to the plant species, explant types, or other factors such as *A. tumefaciens* strain and plasmid. The number of events with a single insert produced using the *A. tumefaciens*-mediated transformation was significantly higher than that with the biolistic method. Using similar constructs, the same cultivar and regeneration and selection protocol, 77 events were produced via the biolistic method in our laboratory. Only 17% (13/77) plants contained single copies of transgenes (data not shown).

The genetic analysis of T_1 and T_2 progeny also provided strong evidence of the incorporation of T-DNA into the wheat genome. The NPT II and GUS genes were inherited to the T_1 and T_2 generations in a Mendelian fashion in most of the events. The data from the Southern analysis of the T_1 generation supported the genetic data in most of the cases, although non-Mendelian segregation patterns were observed occasionally. Similar results were also reported in rice and maize (Hiei et al., 1994; Ishida et al., 1996) and in dicot species transformed by *A. tumefaciens* (Hobbs et al., 1990; Ulian et al., 1994). Gene silence and nondetectable gene expression level in the transgenic plants might be partially responsible for causing the abnormal segregation patterns.

In summary, we have developed a method for rapid production of transgenic plants via *A. tumefaciens* from three kinds of explants of wheat. The transformation efficiency was 0.14 to 4.3% based on the experiments that produced the transgenic plants. The transformed plants appeared to be morphologically identical to nontransformed, growth chamber-grown control plants. In most of the cases, the transformed genes behaved as dominant loci exhibiting normal Mendelian segregation. Therefore, an *A. tumefaciens*-mediated transformation system is now available as an alternative routine method for genetic transformation of wheat.

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LITERATURE CITED

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. Planta **199:** 612–617

Altpeter F, Vasil V, Srivastava V, Stöger E, Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep **16:** 12–17

Becker D, Brettschneider R, Lörz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellum tissue. Plant J **5:** 299–301

Chan M-T, Chang H-H, Ho S-L, Tong W-F, Yu S-M (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. Plant Mol Biol **22:** 491–506

Chan M-T, Lee T-M, Chang H-H (1992) Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. Plant Cell Physiol **33:** 577–583

Cheng M, Jarret RL, Li Z, Xing A, Demski JW (1996) Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens*. Plant Cell Rep **15:** 653–657

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and their progeny. EMBO J **3:** 1681–1689

Deng W-Y, Lin X-Y, Shao Q-Q (1990) *Agrobacterium tumefaciens* can transform *Triticum aestivum* and *Hordeum vulgare* of gramineae. Science in China (Series B) **33:** 27–33

Fry J, Barnason A, Horsch RB (1987) Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. Plant Cell Rep **6:** 321–325

Gould J, Devery M, Hasegawa O, Ulian EC, Peterson G, Smith RH (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol **95:** 426–434

Grimsley NH, Ramos C, Hein T, Hohn B (1988) Meristematic tissues of maize plants are most susceptible to Agroinfection with maize streak virus. Bio/Technology **6:** 185–189

Hess D, Dressler K, Nimmrichter R (1990) Transformation experiments by pipetting *Agrobacterium* into the spikelets of wheat (*Triticum aestivum* L.). Plant Sci **72:** 233–244

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J **6:** 271–282

Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch

RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Bio/Technology **6:** 915–922

Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol Biol **15:** 851–864

Hood EE, Helmer GL, Fraley RT, Chilton M (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacterial **168:** 1291–1301

Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, Hoffmann N (1984) Inheritance of functional genes in plants. Science **223:** 496–498

Horsch RB, Fry JE, Hoffmann N, Eicholz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol **14:** 745–750

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Mooney PA, Goodwin PB, Dennis ES, Llewellyn DJ (1991) *Agrobacterium tumefaciens*-gene transfer into wheat tissues. Plant Cell Tiss Org Cult **25:** 209–218

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Nehra N, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha K (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellum tissues following microprojectile bombardment with two distinct gene constructs. Plant J **5:** 285–297

Ortiz JPA, Reggiardo MI, Ravizzini RA, Altabe SG, Cervigni GDL, Spitteler MA, Morata MM, Elías FE, Vallejos RH (1996) Hygromycin resistance as an efficient selectable marker for wheat stable transformation. Plant Cell Rep **15:** 877–881

Raineri DM, Bottino P, Gordon MP, Nester EW (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L). Bio/Technology 8: 33–38

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Rep **15:** 727–730

Roger SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herharium and mummified plant tissues. Plant Mol Biol 5: 69–76

Schläppi M, Hohn B (1992) Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. Plant Cell **4:** 7–16

Schroeder HE, Schotz AH, Wardley-Richardson T, Spencer D, Higgins TJV (1993) Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). Plant Physiol **101:** 751–757

Shen WH, Escudero J, Schläppi M, Ranos C, Hohn B, Koukolikova-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize tissues. Proc Natl Acad Sci USA **90:** 1488–1492

Songstad DD, Somers DA, Griesbach RJ (1995) Advances in alternative DNA delivery techniques. Plant Cell Tiss Org Cult **40:** 1–15

Ulian EC, Magill JM, Smith RH (1994) Expression and inheritance pattern of two foreign genes in petunia. Theor Appl Genet **88:** 433–440

Umbeck P, Swain W, Yang NS (1989) Inheritance and expression of genes for kanamycin and chloramphenicol resistance in transgenic cotton plants. Crop Sci **29:** 196–201

Vasil V, Srivastava V, Castillo AM, Fromm ME, Vasil IK (1993) Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology **11:** 1553–1558

Weeks JT, Anderson OD, Blechl A (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L). Plant Physiol **102:** 1077–1084

Zhou H, Arrowsmith JW, Fromm ME, Hironaka CM, Taylor ML, Rodriguez D, Pajeau ME, Brown SM, Santino CG, Fry JE (1995) Glyphosate-tolerent CP4 and GOX genes as a selectable marker in wheat transformation. Plant Cell Rep **15:** 159–163

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Transgenic sorghum plants via microprojectile bombardment

(*Sorghum bicolor*/gene transfer/particle gun)

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ABSTRACT **Transgenic sorghum plants have been obtained after microprojectile bombardment of immature zygotic embryos of a drought-resistant sorghum cultivar, P898012. DNA delivery parameters were optimized based on transient expression of *R* and *C1* maize anthocyanin regulatory elements in scutellar cells. The protocol for obtaining transgenic plants consists of the delivery of the *bar* gene to immature zygotic embryos and the imposition of bialaphos selection pressure at various stages during culture, from induction of somatic embryogenesis to rooting of regenerated plantlets. One in about every 350 embryos produced embryogenic tissues that survived bialaphos treatment; six transformed callus lines were obtained from three of the eight sorghum cultivars used in this research. Transgenic (T_0) plants were obtained from cultivar P898012 (two independent transformation events). The presence of the *bar* and *uidA* genes in the T_0 plants was confirmed by Southern blot analysis of genomic DNA. Phosphinothricin acetyltransferase activity was detected in extracts of the T_0 plants. These plants were resistant to local application of the herbicide Ignite/Basta, and the resistance was inherited in T_1 plants as a single dominant locus.**

Sorghum (*Sorghum bicolor* L. Moench) is an important grain and forage crop that is uniquely adapted to semiarid environments. It is typically the cereal grown in areas where the extremes of high temperature and low soil moisture are unsuitable for maize. In 1991 sorghum was fifth in production amongst all cereals with 58 million metric tons harvested on 45 million hectares of land (1). It is a primary staple in the semiarid tropics of Africa and Asia for over 300 million people. These are evidentiary statistics of the significance of this crop in these regions, which are predominated by subsistence agriculture. In the western hemisphere sorghum is used primarily as livestock feed (2). The development of hybrid varieties of sorghum in the 1950s contributed substantially to the increase in production in the United States. Presently, sorghum is third amongst cereals in U.S. production and is the preferred crop in areas of low water availability because of its yield stability under drought conditions.

Advances in biotechnology are now beginning to be used to augment traditional approaches for crop improvement. Restriction fragment length polymorphism (RFLP) linkage maps are being constructed that should greatly facilitate plant breeding efforts for marker-assisted backcross programs (3, 4) and, in the near future, may be used to clone agriculturally important genes through the use of map-based cloning strategies (5). To this date, however, there are no programs in sorghum to access the pool of genes that are available as the result of genetic engineering research, because of the lack of a transformation system. Transformation of protoplasts by electroporation (6) or cell suspensions by microprojectile bombardment (7) has resulted in stable expression of transferred genes; however, transgenic plants were not obtained.

Microprojectile bombardment as a method to introduce DNA into cells circumvents two major constraints of cereal transformation. These are the lack of an available natural vector such as *Agrobacterium tumefaciens* and the difficulty to regenerate plants when protoplasts are used for transformation. Particle bombardment can target cells within tissues or organs that have high morphogenic potential. Immature or mature zygotic embryos (8–10), immature inflorescences (11, 12), and shoot tips (13) of sorghum exhibit embryogenic competence.

In this paper, we describe a procedure to produce transgenic sorghum plants. To date, transformation systems have been described for cereals of major agricultural importance including maize (14, 15), oat (16), rice (17, 18), and wheat (19). Notable exceptions among important cereals in the world are barley and sorghum. Our research has defined parameters for the optimization of DNA delivery to immature zygotic embryos by microprojectile bombardment, identified an appropriate selection agent, bialaphos, and established a selection protocol to recover transformed embryogenic callus and transgenic (T_0) plants. Herbicide resistance was transferred to the T_1 progeny.

MATERIALS AND METHODS

Plant Material and Tissue Culture. Sorghum genotypes representing a range of genetic backgrounds and a variety of agronomic types were used in this research. These included a high-tannin (IS4225), three food grain (CS3541, M91051 and Tx430), and two drought-resistant (P898012, P954035) cultivars, as well as a *Striga*-resistant (SRN39) and a Kaoliang sorghum (Shanqui red) cultivar.

For isolation and culture of immature embryos, the procedure of T. Cai and L.G.B. (unpublished data) was utilized. Immature zygotic embryos (12–15 days after pollination) were isolated and cultured onto a basal medium containing Murashige and Skoog (20) salts, modified B5 vitamins (ref. 21; without the addition of calcium panthotenate) and agar (8 mg/ml; Taiyo bacteriological grade) supplemented with asparagine (150 μg/ml), 10% coconut water, 2,4-dichlorophenoxyacetic acid (2 μg/ml), and sucrose (30 mg/ml) (I_6 medium) for induction of embryogenesis and initiation of embryogenic callus. Procedures for selection and maintenance of embryogenic tissue and shoot and root formation from organized structures were as described (12). Media contained the basal constituents supplemented with 2,4-dichlorophenoxyacetic acid (2 μg/ml), kinetin (0.5 μg/ml), sucrose (30 mg/ml) for maintenance of embryogenic tissue or indole-3-

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Abbreviations: GUS, β-glucuronidase; PAT, phosphinothricin acetyltransferase.

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acetic acid (1 μg/ml), kinetin (0.5 μg/ml), and sucrose (20 mg/ml) to facilitate shoot development. Plantlets (about 2 cm in height) were transferred to medium containing Murashige and Skoog salts (all at 0.5 × concentration), 1-naphthaleneacetic acid (0.5 μg/ml), indole-3-butyric acid (0.5 μg/ml), sucrose (20 mg/ml), and agar (8 mg/ml) to allow root development. Bialaphos and indole-3-acetic acid were filter (0.2-μm pore diameter) sterilized and added to cooled media (40–50°C). Cultures of immature embryos and embryogenic tissue were grown in darkness and recultured every 2 weeks and were maintained at 26°C. Tissues on shoot regeneration medium were subcultured every 4 weeks and grown at 26°C under a 16-hr photoperiod (1000–2000 lx from fluorescent, cool white light).

Plasmids. The plasmids used in this research, pPHP620 (8.537 kb) and pPHP687 (9.056 kb), were provided by Pioneer Hi-Bred International. In these plasmids (pUC18) all marker genes were driven by a double cauliflower mosaic virus 35S promoter (22), with the ΩRNA leader sequence (23) and the first intron of the maize alcohol dehydrogenase gene (24). The plasmid pPHP620 contains the reporter gene *uidA* (25), encoding β-glucuronidase (GUS), and the selectable marker gene *bar* (26), encoding phosphinothricin acetyltransferase (PAT). pPHP687 contains the maize anthocyanin regulatory elements *R* (27) and *C1* (28).

Microprojectile Bombardment. All experiments were conducted with the Biolistics PDS 1000/He system (29) using tungsten (M-25, 1.7 μm in diameter, DuPont no. 75056) or gold (1.5–3.0 μm in diameter, Aldrich no. 32,658-5) microprojectiles. Gold (3 mg) or tungsten (0.75 mg) particles (previously washed in ethanol) in aqueous suspension (50 μl) were coated with 5–10 μg of plasmid DNA, as described by the manufacturer (Bio-Rad). The particles were finely dispersed with an ultrasonic cleaner (Sonicor Instrument Corporation, Copiague, NY) before bombardment. Bombardment pressures and distances from the launching plate were experimentally determined and were as indicated in the text. The plasmids were mixed in a ratio of 5 μg of pPHP620 to 1 μg of pPHP687—i.e., 1 μg/0.2 μg—per bombardment, or pPHP620 was used alone at 2 μg per bombardment.

Immature zygotic embryos, 10–15 per plastic Petri dish (15 × 60 mm, Falcon no. 1007), were bombarded between 24 and 72 hr after culture onto I_6 medium. The embryos were transferred onto filter papers (4.5 cm in diameter; Baxter glass fiber no. 391 overlayed onto Whatman no. 1) that were premoistened but not saturated with liquid I_6 medium. The filter papers functioned to absorb the water from the surface of the embryos, and the embryos were left for 2–3 hr on the papers prior to bombardment. Immediately after bombardment, the immature embryos were removed from the papers and transferred to semisolid I_6 medium.

Selection Agent. The herbicide bialaphos (Meiji Seika, Yokohama, Japan) was used as the selection agent in these experiments (30). Bialaphos was dissolved in water (0.1 g/ml) and purified by filtration through a C_{18} reverse-phase column (Baker no. 7020-13). The filtrate was diluted to a final bialaphos concentration of 1 mg/ml.

Analysis of Transgenic Tissues and Plants. Transient expression was evaluated 48 hr after bombardment. First, anthocyanin accumulation (31) was determined. GUS activity was then evaluated by histochemical assay (25). The number of red (anthocyanin accumulation) or blue (GUS activity) spots was determined using a stereo microscope (×50 magnification). Each distinct spot was counted as one expression event and the results are expressed as mean number of spots per embryo ± SE. Control embryos were bombarded with particles without DNA and no anthocyanin or GUS activity was detected. Samples for histology were prepared as described by Kononowicz *et al.* (32).

For DNA blot analysis, 15 μg of genomic DNA was isolated according to Dellaporta *et al.* (33), reacted overnight with restriction endonucleases, separated in 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P labeled probe (34). The *bar* gene was detected with a 0.8-kb fragment from pPHP620 that contains the entire *bar* coding region and the proteinase inhibitor II (PinII) terminator, whereas the *uidA* gene was visualized with a 2.2-kb fragment corresponding to the GUS coding region and the nopaline synthase terminator.

PAT activity was evaluated in callus and leaf extracts according to DeBlock *et al.* (30). The effects of the herbicide were assessed 2 and 4 days after local application of a 0.6% aqueous solution of Ignite/Basta (Hoechst), containing 0.1% Tween 20 onto the surface of young leaves. This herbicide contains glufosinate (200 mg/ml), the ammonium salt of phosphinothricin. T_1 seedlings (two- or three-leaf stage) were sprayed with the herbicide and survival was evaluated 5 days later.

RESULTS

Optimization of DNA Delivery Parameters by Transient Expression. The maize *R* and *C1* transcriptional activators appeared to function in cells of immature zygotic sorghum embryos (Fig. 1*A*). Since anthocyanin accumulation is cell autonomous and its visualization is nondestructive, it was chosen as the marker to optimize DNA delivery to individual cells in the scutellum of immature embryos. Little difference was detected based on the type of microprojectile used. A substantially more uniform microprojectile bombardment pattern and greater reporter gene expression was achieved when the microcarriers were dispersed with an ultrasonic cleaner prior to loading onto the flying disk (Fig. 1 *A* and *B*). GUS expression was detected 4 and 15 days after bombardment indicating that division and growth of the cells to which DNA was delivered had occurred (Fig. 1 *C* and *D*).

Cultured sorghum cells release phenolics into the culture medium (Fig. 2*A*), and the oxidized products inhibited morphogenesis and growth (8) and interfered with the evaluation

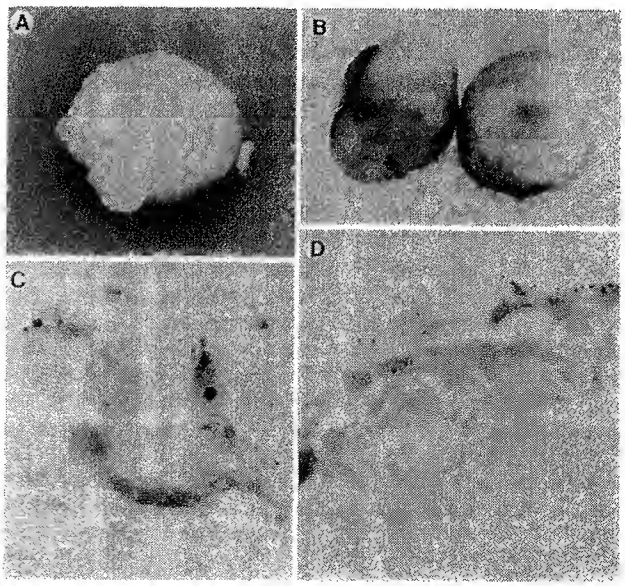

FIG. 1. Transient gene expression in the scutellum of immature zygotic sorghum embryos after DNA delivery by particle bombardment. Anthocyanin accumulation (*A*) and GUS activity (*B*) are detected 48 hr after bombardment. A transverse section of the scutellum (*C*) shows GUS activity 4 days after bombardment. GUS activity is seen in callus derived from the scutellum, 15 days after bombardment (*D*). (*A* and *B*, ×10; *C*, ×300; *D*, ×150.)

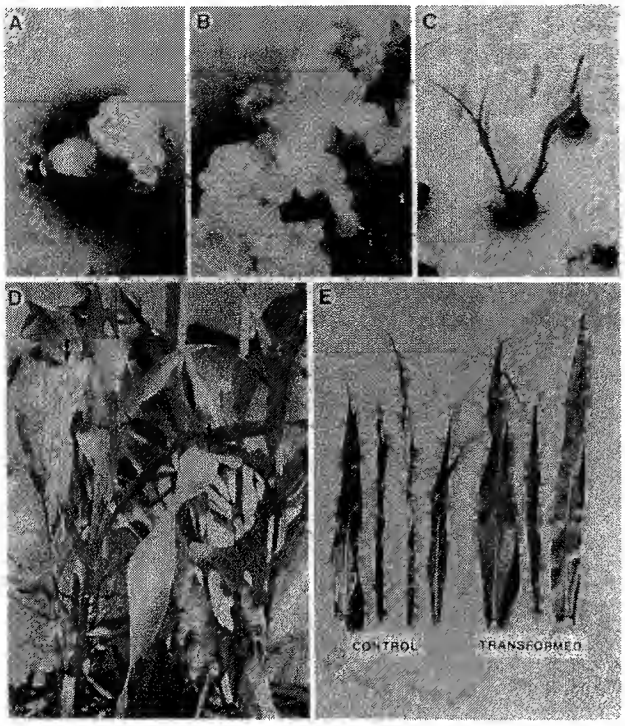

FIG. 2. Selection of transformed sorghum (cultivar P898012) callus, plant regeneration, and herbicide resistance of transgenic plants. (*A*) Immature zygotic embryo on induction medium containing bialaphos at 1 μg/ml. (*B*) Embryogenic callus on maintenance medium with bialaphos at 3 μg/ml. (*C*) Plant regeneration on medium with bialaphos at 3 μg/ml. (*D*) Untransformed (left) and transformed (right) regenerated plants 48 hr after local application of herbicide to the leaves (arrows). (*E*) Leaves from four untransformed control plants (from the left, nos. 1 and 2 were seed derived, and nos. 3 and 4 were regenerated) and three transformed plants, 4 days after treatment with the herbicide.

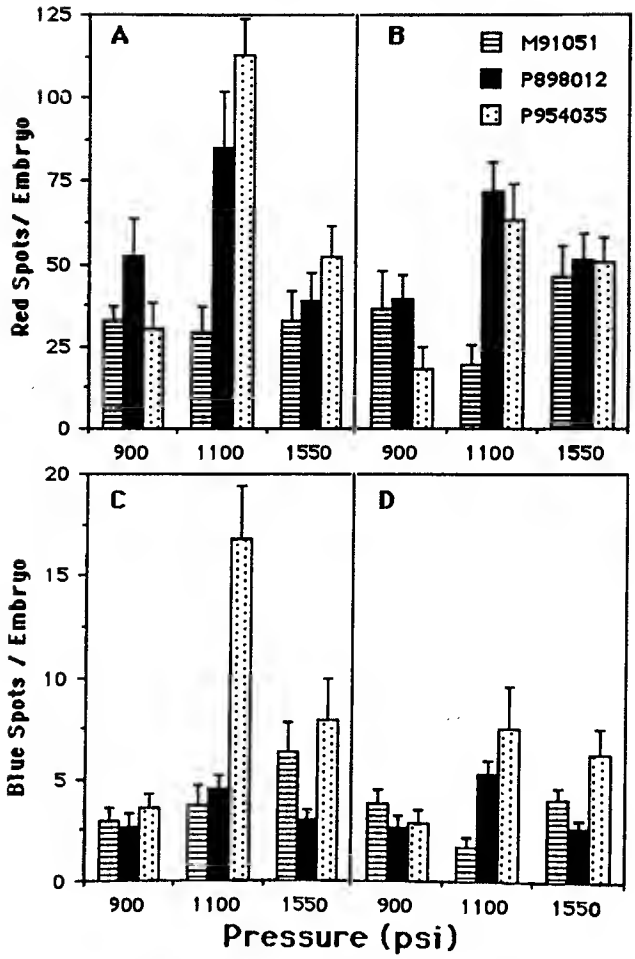

FIG. 3. Transient expression of *R* and *C1* transcriptional activators and the *uidA* gene in immature zygotic sorghum embryos. Anthocyanin accumulation (*A* and *B*) and GUS activity (*C* and *D*) were evaluated in three cultivars (M91051, P898012, and P954035), at three bombardment pressures (pressure) and at two distances between the embryos and the particle launch site (*A* and *C*, 6.0 cm; *B* and *D*, 9.2 cm).

of *R* and *C1* expression. The moistened glass fiber filter and filter paper support onto which embryos were placed for bombardment absorbed the surface moisture from tissues. This presumably facilitated increased particle penetration and higher transformation frequencies. It was repeatedly observed that bombardments conducted when embryos were on the support resulted in a higher percentage of embryos expressing the introduced genes (data not shown). Further, there was less phenolic pigment production during the subsequent culture period.

DNA delivery was estimated by transient expression of the regulatory elements *R* and *C1*, but these genes are in a different plasmid than *uidA* and *bar* (Fig. 1 *A* and *B*). Although differences in the absolute expression of *R/C1* and *uidA* genes were detected, similar patterns of relative expression as a function of genotype, bombardment pressure, and distance were observed (Fig. 3). The levels of expression of both reporter genes detected in sorghum tissues were lower than those detected in maize type I callus (data not shown). The greatest effect on reporter gene expression was attributable to variation amongst genotypes; however, there also appeared to be a genotype by bombardment pressure interaction (Fig. 3).

Selection of Transformed Callus and Plant Regeneration. Sorghum tissues were very sensitive to bialaphos. Concentrations greater than 3 μg/ml for a 4-week exposure period were toxic. Different selection strategies were imposed on immature embryos during the induction stage to accommodate the possibility that immediate exposure to bialaphos would be lethal to the transformed cells. Embryos were grown on induction medium without selection for 2 weeks and then transferred to maintenance medium with bialaphos at 3 μg/ml, or selection was started on induction medium by transferring the embryo immediately after bombardment or 3 or 7 days later to bialaphos at 1 μg/ml. Once that embryogenic callus had developed, tissues were transferred to maintenance medium with the herbicide at 3 μg/ml. Although substantial cell death occurred on induction medium supplemented with bialaphos (Fig. 2*A*), no clear difference could be attributed to the different selection strategies. However, all the material that was transferred directly to high bialaphos eventually died. Transfer of calli to maintenance medium with the herbicide at 3 μg/ml imposed much higher selection pressure on the tissues. Since GUS activity could not be detected in sorghum tissues later than 3 weeks after bombardment, growth on bialaphos at a rate similar to untransformed tissue on medium without bialaphos was used as an initial phenotypic indicator of transformed callus (Fig. 2*B*).

Embryogenic calli that had survived for 3 months on medium with bialaphos at 3 μg/ml were transferred to regeneration medium containing the same level of bialaphos and grown under light. A high proportion of this callus remained viable, whereas callus from embryos not bombarded with the *bar*-containing plasmid died within 2 weeks. Plantlets (Fig. 2*C*) were maintained on regeneration medium for 2–3 months and then rooted on medium with bialaphos at 1 μg/ml. The regenerated plants were transferred to soil,

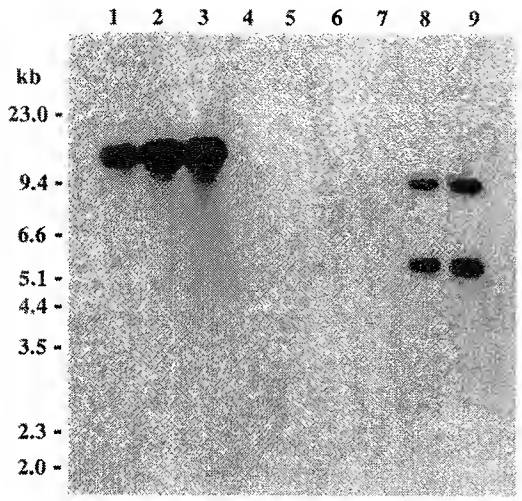

FIG. 4. Southern blot analysis of genomic DNA from transgenic sorghum plants (cultivar P898012). Plant genomic DNA (15 μg) was digested with *Eco*RI and probed with the *bar* insert. DNA was from plants regenerated from transformed callus line 1119 (lanes 1–3), untransformed seed-derived (lane 4) and regenerated (lane 5) plants of cultivar P898012, untransformed seed-derived (lane 6) and regenerated (lane 7) plants of cultivar P954035, and plants regenerated from transformed callus line 1409 (lanes 8 and 9).

acclimated to low humidity in a growth chamber, and eventually moved to the greenhouse. The total period from initiation of the cultures to acclimation of plants in the greenhouse was 7 months.

Most of the putative transformed calli that survived bialaphos treatment on maintenance medium died when transferred to bialaphos-containing regeneration medium. Of the surviving calli only a few exhibited shoot development. Exposure to light accelerates the development of phytotoxic activity of the herbicide. These results suggest that the calli might not be uniformly transformed and that only the cell sectors expressing the *bar* gene survive and grow after being transferred to the light.

Only three of the eight genotypes (cultivars P898012, P954035, and Tx430) produced embryogenic calli that survived the bialaphos selection strategies imposed in this study. Approximately 1 out of 350 embryos (6 out of 2150 total) that were bombarded produced calli that survived bialaphos. Calli of only the cultivar P898012 regenerated plants after being subjected to bialaphos selection pressure on the regeneration and root-formation media. The lack of success in recovering embryogenic callus of cultivars CS3541, M91051, SRN39, and Shanqui red that survives bialaphos selection may be partially attributable to their low morphogenic response from

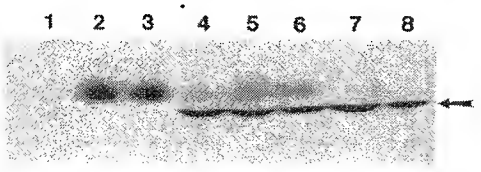

FIG. 5. PAT activity in leaves of transgenic sorghum (cultivar P898012) plants regenerated from callus lines 1119 and 1409. The reaction mixture contained 30 μg of protein in 24 μl of a buffer solution (50 mM Tris·HCl, pH 7.5/2 mM EDTA) plus 4 μl of 1 mM phosphinothricin and 4 μl of acetyl-^{14}C-CoA (sp. act. 57.9 mCi/mmol; NEN). Lanes 1, no plant extract; lanes 2 and 3, extracts from untransformed seed-derived and regenerated plants, respectively; lanes 4–6, extracts from three plants from line 1119; lane 7, extract from regenerated plant from line 1409; lane 8, extract from transgenic rice plant (35). Sixteen microliters (lanes 1–7) or 8 μl (lane 8) was applied to the chromatogram. Acetylated phosphinothricin is identified with the arrow.

immature embryos. These genotype-related differences might be diminished by using different explants (immature inflorescences or shoot tips; unpublished data) as targets for transformation.

Evaluation of Transgenic Material. Embryogenic calli from two immature embryos of cultivar P898012 (1119 and 1409) survived bialaphos selection and produced plants. Genomic DNA hybridization with the *bar* probe indicated the presence of one and two genes in plants regenerated from lines 1119 and 1409, respectively (Fig. 4). The restriction enzyme used linearized the plasmid pPHP620 in a region flanking the *bar*–PinII probe. The presence of distinctive bands required digestion of the plant DNA, demonstrating integration of the gene in the plant genome. Analogous results were obtained with the *uidA* probe (data not shown).

The plants regenerated from the two callus lines were morphologically similar to seed-derived sorghum plants. Plants derived from callus line 1119 flowered and all of them were fully fertile. These plants were resistant to bialaphos (Fig. 2 *D* and *E*), and this resistance was due to the expression of the *bar* gene (Fig. 5). One plant regenerated from the 1409 line has been evaluated for PAT activity and the result was positive (Fig. 5). The leaves of control plants (seed-derived or regenerated from tissues bombarded without DNA) treated with Ignite/Basta suffered substantial necrosis within 48 hr and eventually died. In many cases, the symptoms spread to the surrounding leaves, leading to the death of the entire tiller.

Analysis of the T_1 progeny showed segregation for herbicide resistance with 97 of 129, 145 of 182, and 52 out of 69 seedlings (derived from three plants regenerated from callus line 1119) surviving after treatment with the herbicide, whereas all control seedlings died. These results agree with a 3:1 Mendelian segregation for a single dominant locus ($P > 0.05$).

DISCUSSION

Transgenic sorghum plants have been obtained following microprojectile bombardment of immature zygotic embryos. Delivering DNA directly to the primary explant results in transformation of the embryogenically competent cells in the scutellum prior to the initiation of embryogenesis (10). Regardless of whether the somatic embryos arise directly from competent cells or are derived from a callus intermediary (36), DNA is delivered at a stage when the one (or few) cell progenitor of each organized structure exists. This should reduce or eliminate chimerism (the mixture of untransformed and transformed cells) in the organized structure or regenerated plants.

Transgenic plants were obtained from only 2 of 600 embryos of cultivar P898012 that were initially subjected to microprojectile bombardment. Although the reason for this is not clear at this point, the low frequency may be attributable to a number of different factors that affect the transformation process. It is generally assumed that transient expression is an indicator of the potential for DNA integration and stable expression. Although the DNA delivery parameters were defined as a part of this research, it is possible that an optimal protocol has yet to be established. The transformation vectors were designed for use with maize, and perhaps these constructs are not optimal for expression in sorghum cells. Transient expression in sorghum is lower than in maize. It is also possible that inherent characteristics of the sorghum scutellar cells make them somewhat unresponsive for transient expression. Osmotic pretreatments have been shown to increase transient expression and enhance integration of transferred DNA, and perhaps these would increase the responsiveness of sorghum cells (37, 38).

Evidence also indicates that the transferred gene(s) is not expressed after sustained periods of culture. GUS activity,

which was high in transient assays, could not be detected in callus that had been maintained for prolonged periods on bialaphos selection pressure, despite the fact that Southern analysis indicated the presence of the *uidA* gene. This suggests that DNA methylation occurs in sorghum cells that inactivates the expression of transferred genes (39, 40).

Although *bar* has proven to be a reliable selectable marker gene, conditions have not been established to optimize the application of bialaphos selection pressure for the efficient recovery of transgenic plants. The impact of selection pressure at various stages of embryogenic differentiation and shoot and root development needs to be established. Assuming some efficient capacity to screen for transgenic plants, it is not certain that applying selection pressure to the extent that all nontransformed cells or organized structures are eliminated is necessarily the most effective way to obtain transgenic plants.

The sorghum cultivar transformed, P898012, is well adapted in Niger and Sudan and is known to have both preflowering and postflowering drought resistance. The availability of a gene transformation and regeneration system in sorghum opens up new opportunities to improve protein nutritional quality and other traits of a high-yield, drought-resistant sorghum cultivar which serves as a staple food for millions of people in sub-Saharan Africa.

We have now demonstrated the usefulness of a selectable marker gene to optimize a sorghum transformation system which will eventually allow the introduction of agronomically important traits to sorghum by genetic transformation.

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1. Food and Agriculture Organization of The United Nations (1992) *FAO Production Yearbook 1991* (FAO, Rome), Vol. 45.
2. Rooney, L. W. & Serna-Saldivar, S. O. (1991) in *Handbook of Cereal Science and Technology*, eds. Lorenz, K. J. & Kulp, K. (Marcel Decker, New York), pp. 233–267.
3. Whitkus, R., Doebley, J. & Lee, M. (1992) *Genetics* **132**, 1119–1130.
4. Melake Berhan, A., Hulbert, S. H., Butler, L. G. & Bennetzen, J. L. (1993) *Theor. Appl. Genet.* **86**, 598–604.
5. Martin, G. B., de Vicente, M. C. & Tanksley, S. D. (1993) *Mol. Plant-Microbe Interact.* **6**, 26–34.
6. Battraw, M. & Hall, T. C. (1991) *Theor. Appl. Genet.* **82**, 161–168.
7. Hagio, T., Blowers, A. D. & Earle, E. D. (1991) *Plant Cell Rep.* **10**, 260–264.
8. Cai, T., Daly, B. & Butler, L. (1987) *Plant Cell Tissue Organ Cult.* **9**, 245–252.
9. Dunstan, D. I., Short, K. C., Dhaliwal, H. & Thomas, E. (1979) *Protoplasma* **101**, 355–361.
10. Dunstan, D. I., Short, K. C. & Thomas, E. (1978) *Protoplasma* **97**, 251–260.
11. Brettell, R. I. S., Wernicke, W. & Thomas, E. (1980) *Protoplasma* **104**, 141–148.
12. Cai, T. & Butler, L. (1990) *Plant Cell Tissue Organ Cult.* **20**, 101–110.
13. Bhaskaran, S. & Smith, R. H. (1988) *In Vitro Cell. Dev. Biol.* **24**, 65–70.
14. Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. & Klein, T. M. (1990) *Bio/Technology* **8**, 833–839.
15. Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Jr., Willetts, N. G., Rice, T. B., Mackey, C. J., Krueger, R. W., Kausch, A. P. & Lemaux, P. G. (1990) *Plant Cell* **2**, 603–618.
16. Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F. & Bushnell, W. R. (1992) *Bio/Technology* **10**, 1589–1594.
17. Cao, J., Duan, X., McElroy, D. & Wu, R. (1992) *Plant Cell Rep.* **11**, 586–591.
18. Christou, P., Ford, T. L. & Kofron, M. (1991) *Bio/Technology* **9**, 957–962.
19. Vasil, V., Castillo, A. M., Fromm, M. E. & Vasil, I. K. (1992) *Bio/Technology* **10**, 667–674.
20. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15**, 473–497.
21. Gamborg, O. L., Shyluk, J. P., Brar, D. S. & Constabel, F. (1977) *Plant Sci. Lett.* **10**, 67–74.
22. Kay, R., Chan, A., Daly, M. & McPherson, J. (1987) *Science* **236**, 1299–1302.
23. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids Res.* **15**, 3257–3272.
24. Callis, J., Fromm, M. & Walbot, V. (1987) *Genes Dev.* **1**, 1183–1200.
25. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) *EMBO J.* **6**, 3901–3907.
26. Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M. & Botterman, J. (1987) *EMBO J.* **6**, 2519–2523.
27. Ludwig, S. R., Habera, L. F., Dellaporta, S. L. & Wessler, S. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7092–7096.
28. Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P. A. & Saedler, H. (1987) *EMBO J.* **6**, 3553–3558.
29. Sanford, J. C., Devit, M. J., Russell, J. A., Smith, F. D., Harpending, P. R., Roy, M. K. & Johnston, S. A. (1991) *Technique J. Methods Cell Mol. Biol.* **3**, 3–16.
30. DeBlock, M. E., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N. R., Thompson, C., Van Montagu, M. & Leemans, J. (1987) *EMBO J.* **6**, 2513–2518.
31. Ludwig, S. E., Bowen, B., Beach, L. & Wessler, S. R. (1990) *Science* **247**, 449–450.
32. Kononowicz, A. K., Nelson, D. E., Singh, N. K., Hasegawa, P. M. & Bressan, R. A. (1992) *Plant Cell* **4**, 513–524.
33. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1**, 19–21.
34. Sambrook, J., Fritsch, E. F. & Maniatis, T., eds. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
35. Rathore, K. S., Chowdhury, V. K. & Hodges, T. K. (1993) *Plant Mol. Biol.* **21**, 871–884.
36. Vasil, V., Lu, C.-Y. & Vasil, I. K. (1985) *Protoplasma* **127**, 1–8.
37. Russell, J. A., Roy, M. K. & Stanford, J. C. (1992) *In Vitro Cell. Dev. Biol.* **28P**, 97–105.
38. Vain, P., McMullen, M. D. & Finer, J. J. (1993) *Plant Cell Rep.* **12**, 84–88.
39. Klein, T. M., Kornstein, L. & Fromm, M. E. (1990) in *Gene Manipulation in Plant Improvement II*, ed. Gustafson, J. P. (Plenum, New York), pp. 265–288.
40. Bochardt, A., Hodal, L., Palmgren, G., Mattsson, O. & Okkels, F. T. (1992) *Plant Physiol.* **99**, 409–414.

Transformation of Oat Using Mature Embryo-Derived Tissue Cultures

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ABSTRACT

Mature embryos of oat (*Avena sativa* L.) have been used to establish regenerable tissue cultures with potential use for transformation. The objective of this study was to investigate tissue cultures established from mature embryos of oat as an alternative source of totipotent target cells for microprojectile bombardment-mediated transformation. Mature embryos of a specific genotype, GAF/Park-1, were incubated on a tissue culture induction medium for 1, 4, 8, or 9 wk before either being directly bombarded after 1 and 4 wk, or bombarded as tissue cultures initiated after 8 and 9 wk incubation. The 8- and 9-wk-old tissue cultures yielded the greatest numbers of transgenic tissue cultures (3.2 transgenic tissue cultures per microprojectile bombardment treatment). Three additional transformation experiments were conducted with mature embryo-derived 8- to 9-wk-old tissue cultures to determine the regeneration capacity and production of fertile transgenic plants. Overall, fertile plants were regenerated from 35 of 85 independently derived transgenic tissue cultures. Identification of mature embryo-derived tissue cultures as a source of transformable totipotent cells should reduce the expense and labor involved in oat transformation. Moreover, the uniformity and convenience of this explant likely will stimulate further investigations in oat transformation efficiency.

OAT TRANSFORMATION has utilized friable, embryogenic callus initiated from immature embryos as a source of totipotent target cells for microprojectile bombardment (Somers et al., 1992, 1996; Torbert et al., 1995). This transformation system yields fertile, transgenic plants, but has certain disadvantages primarily because it utilizes immature embryo-derived tissue cultures. For example, initiation of friable, embryogenic callus requires growing plants to 12 to 18 d post anthesis under optimal conditions for embryo isolation and at least 12 wk for callus initiation and growth before microprojectile bombardment. Growth of donor plants is expensive, and immature embryo isolation is laborious; thus, the tissue cultures often are used for up to 1 yr because of the investment in their establishment. Furthermore, we have observed reduced plant regeneration and fertility of regenerated transgenic plants to be associated with tissue culture age and suspect that somaclonal variation is responsible for these reductions. In oat plants regenerated from tissue culture, the frequency of chromosomal abnormalities varies among genotypes and increases with the age of the tissue cultures (McCoy et al., 1982; Rines et al., 1986). Because chromosomal and genetic changes induced by the tissue culture process likely would result in reduction of totipotency in tissue culture cells and of fertility in regenerated plants, any method that shortens the tissue culture period required to produce transgenic plants may result in improved production of fertile, transgenic oat plants. Consequently, we have attempted to minimize the age of the tissue cultures used to conduct microprojectile bombardment. However, reduction of tissue culture age requires growth of donor plants year round for initiation of greater numbers of short-term tissue cultures from immature embryos, further increasing the labor and expense associated with production of fertile, transgenic plants (Sivamani et al., 1996). Finally, initiation of friable, embryogenic tissue cultures from immature embryos in oat is most efficient with a specific genotype, GAF/Park (Cummings et al., 1976; Bregitzer et al., 1989; Gana et al., 1995). Some success in establishment of friable, embryogenic tissue cultures from immature embryos of other cultivars has been reported (Bregitzer et al., 1995). However, there remains a need to identify tissue culture methods that enable transformation of a broader range of genotypes. These observations indicate the requirement for a more convenient explant for routine tissue culture establishment in oat that does not require frequent growth of nearly mature plants, involves short periods in tissue culture, and ideally is not restricted to specific genotypes to usefully apply genetic engineering to oat improvement.

A strategy to reduce tissue culture age used in transformation of rice (*Oryza sativa* L.) (Christou et al., 1991; Cooley et al., 1995), wheat (*Triticum aestivum* L.) (Weeks et al., 1993; Vasil et al., 1993; Nehra et al., 1994), and barley (*Hordeum vulgare* L.) (Wan and Lemaux, 1994) is to conduct microprojectile bombardment of the scutella of mature or immature embryos. In these species, transformable callus originates from the scutellum of the embryo. The most widely used explant for establishment of regenerable tissue cultures of oat is the immature embryo (Cummings et al., 1976; King et al., 1986; Lorz et al., 1976; Rines and McCoy, 1981). However, direct bombardment of oat immature embryos would be impractical because callus initiation arises from the mesocotyl of the immature embryo (Rines and McCoy, 1981; Bregitzer et al., 1989; 1995), a very small target for microprojectile bombardment. In oat, alternative explants, such as mature embryos (Cummings et al., 1976; Heyser and Nabors, 1982; Nabors et al., 1982, 1983), leaf base of growing seedlings (Chen et al., 1995a,b; Gless et al., 1996), apical meristems (A. Nassuth and I. Altosaar, 1989, Personal communication), roots (Nabors et al., 1982), and mesocotyls from mature embryos (Heyser and Nabors, 1982; Bregitzer et al., 1989) have been shown to be capable of

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Abbreviations: BAP, benzylaminopurine; ELISA, enzyme-linked immunosorbent assay; GUS, β-glucuronidase; MS, Murashige and Skoog; NAA, naphthalene acetic acid; NPT II, neomycin phosphotransferase II; *npt II*, gene coding for NPT II; NOS, nopaline synthase; 2,4-D, 2,4-dichlorophenoxyacetic acid; *uid A*, *E. coli* gene coding for GUS; X-gluc, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt.

regenerable tissue culture establishment. In none of these cases, aside from preliminary results with leaf base-derived callus (Gless et al., 1996), have the tissue cultures been used for transformation. The goal of this study was to identify a more convenient explant than the immature embryo for establishment of transformable tissue cultures of oat. Based on review of the oat tissue culture literature (Rines et al., 1992; Bregitzer et al., 1995), mature embryos were chosen as an alternative explant for establishing tissue cultures for transformation experiments. The objectives of this study were to (i) investigate the mature embryo as an explant for regenerable tissue culture initiation and (ii) determine whether mature embryo-derived tissue cultures can be transformed and used to produce fertile, transgenic plants.

MATERIALS AND METHODS

Seed Source

The oat genotype evaluated was GAF/Park-1, a reselection from GAF/Park which is a line selected specifically for high tissue culture response (Rines and Luke, 1985; Bregitzer et al., 1989). Seed increases of GAF/Park-1 were kindly produced by Darrell Wesenberg at the USDA Small Grains Germplasm Research Facility in Aberdeen, ID, where the semi-arid climate retards fungal pathogen and saprophyte development, providing a high quality uniform seed source.

Embryo Isolation and Tissue Culture Initiation

Dehulled oat seed were surface sterilized for 30 s in 95% v/v ethanol, 5 min in a solution of 2.5% w/v sodium hypochlorite (NaOCl) containing 1 to 2 drops of Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma Chemical Co., St. Louis, MO)[1], and rinsed three times in sterile double deionized water for 5 min/rinse. Approximately 100 sterilized seeds were placed in a 125-mL Erlenmeyer flask containing 50 mL sterile double deionized water and incubated on a gyrotory shaker at 145 rpm in the dark at 28°C overnight.

Mature embryos were excised and placed scutellum side down on MS2D medium containing MS salts (Murashige and Skoog, 1962), 150 mg L^{-1} asparagine, 0.5 mg L^{-1} thiamine HCl, 20 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, adjusted to pH 5.8, and solidified with 0.2% w/v Gelrite. The embryos were incubated on the same plate for up to 9 wks in the dark at 20°C. Shoots and roots were excised as they appeared during the first 2 to 4 wk of the culture period. Similar procedures are used for establishing tissue cultures from immature embryos (Bregitzer et al., 1995). After 8 to 9 wk, the embryogenic appearance of the callus was assessed visually by means of a binocular dissecting microscope.

Plasmids, DNA Preparation, and Microprojectile Bombardment

Two plasmids, pNGI and pH24, both carrying the selectable marker neomycin phosphotransferase (*npt II*) conferring resistance to paromomycin (Torbert et al., 1995) were used for different experiments in this study. pNGI contains both the *uid A* (β-glucuronidase, GUS) reporter and *npt II* plant selectable marker genes (Klein et al., 1989). The *uid A* gene is fused to the maize alcohol dehydrogenase I promoter which regulates *uid A* gene expression and therefore GUS activity in transgenic tissue cultures and mature oat groats (Somers et al., 1992). pH24 contains only the *npt II* gene. Gold particles were coated with plasmid DNA using procedures detailed in the Biolistic PDS-1000/He Particle Delivery System manual (BioRad Laboratories, Hercules, CA).

Mature embryos 1 and 4 wk after plating and tissue cultures initiated from mature embryos 8 and 9 wk after plating were transferred onto solid MS2D medium containing 0.2 *M* sorbitol and 0.2 *M* mannitol as an osmoticum pretreatment (Vain et al., 1993) 4 h before microprojectile bombardment. Approximately 0.6 g of embryo explants or tissue culture were placed in the center of a Petri plate forming a 2- to 3-cm-diam. circle. Petri plates were positioned 8.0 cm below the stopping disk and tissue was bombarded with the PDS-1000/He Particle Delivery System. Following microprojectile bombardment, tissue remained on the osmoticum medium overnight and was transferred to MS2D medium for 7 d incubation at 20°C in the dark. Selection for paromomycin-resistant tissue cultures was conducted according to Torbert et al. (1995).

Selection of Transgenic Tissue Cultures and Plant Regeneration

After 8 to 10 wk on selection medium, paromomycin-resistant colonies were placed on regeneration medium (Bregitzer et al., 1989) containing 50 mg L^{-1} paromomycin sulfate. After 4 to 6 wk, shoots were transferred to Magenta boxes containing hormone-free MS medium, without the selection agent, for root formation. After 1 to 2 wk, rooted plants were transferred to Fison's (Bellevue, WA) LC1 potting mix wetted with 1/10th strength MS salts. Plants were placed in growth chambers under conditions described by Torbert et al. (1995) and grown to maturity.

Characterization of Transgenic Phenotypes

The *npt II* gene product was detected in callus and leaf tissues using an NPT II ELISA kit (5'->3', Inc., Boulder, CO). In a previous study (Torbert et al., 1995), the NPT II ELISA kit was used to investigate the transgenic phenotype of oat tissue cultures and plants transformed with the plasmid pNGI. From a sample of 88 NPT II ELISA positive tissue cultures, 29 were randomly selected and analyzed by Southern blots as described in Torbert et al. (1995). All 29 tissue cultures exhibited the unit-length *npt II* gene indicating that the NPT II ELISA is a reliable indicator for transgenic oat.

As additional evidence that the regenerated plants produced were transgenic, β-glucuronidase (GUS) histochemical staining was conducted on tissue cultures and seed produced on plants regenerated from tissue cultures transformed with pNGI. The GUS stain solution was prepared at pH 8.0 and contained 20% v/v methanol to reduce background staining (Jefferson, 1987; Kosugi et al., 1990). Up to 10 seed harvested from regenerated plants were dehulled and cut in half with a scalpel. The endosperm portion was stained for GUS activity. Regenerated plants producing seed that exhibited GUS staining were considered transgenic.

RESULTS

Tissue Culture Response of Mature Embryos

Mature embryos of GAF/Park-1 exhibited a callus initiation frequency of about 50% 8 to 9 wk after explant

[1] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture or the University of Minnesota, and does not imply its approval to the exclusion of other products or vendors which may also be suitable.

isolation. The level of differentiation among the tissue cultures initiated from different mature embryos was variable ranging from highly organized to highly embryogenic (Fig. 1A), and in some cases, the callus appeared nonregenerable. This result was expected at this early stage of culture because no selection for embryogenic callus type had been conducted. Overall, about 30% of the mature embryo-derived tissue cultures resembled in appearance the tissue cultures initiated from immature embryos. Somatic embryoids were present and the callus was friable (Fig. 1A). This frequency with mature embryos was somewhat lower than the 65% embryogenic callus initiation frequency reported from immature embryos (Bregitzer et al., 1989; 1995). The average fresh weight of the 8- to 9-wk-old tissue culture produced per mature embryo was 0.6 g with a range of 0.3 to 0.7 g.

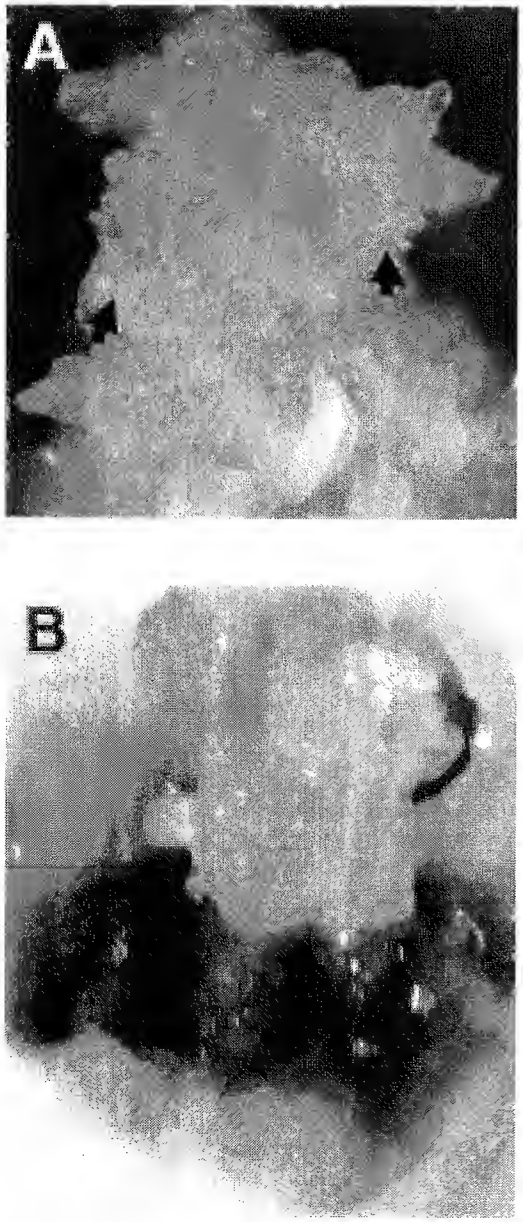

Fig. 1. A) Embryogenic callus initiated from mature oat embryos (arrows indicate some of the regions of somatic embryogenesis). B) Actively growing, putatively transgenic region of a tissue culture on selection medium 6 wk after bombardment.

Transformation of Mature Embryo-Derived Tissue Cultures

A preliminary microprojectile bombardment experiment was conducted to determine the age after embryo plating at which the mature embryo-derived tissue cultures were transformable. The plasmid pNGI was used. Mature embryos were incubated 1, 4, 8, and 9 wk before being either directly bombarded, as in the case of the 1- and 4-wk-old explants, or bombarded as tissue cultures isolated from the 8- and 9-wk-old cultures (Table 1). No paromomycin-resistant tissue cultures were recovered from microprojectile bombardment of the 1-wk-old explants. A total of 73 paromomycin-resistant tissue cultures were produced from the other three treatments. The appearance of paromomycin-resistant tissue cultures selected from bombardment of mature embryo-derived tissue cultures (Fig. 1B) was similar to our previous observations from immature embryo-derived tissue cultures (Torbert et al., 1995). All paromomycin-resistant tissue cultures exhibited the *npt II* gene product as determined by NPT II ELISA. Fifty of the 73 paromomycin-resistant tissue cultures also were GUS positive, which was similar to previously reported coexpression frequencies determined from pNGI oat transformants (Torbert et al., 1995). When the number of transgenic tissue cultures was expressed on the basis of microprojectile bombardment treatments, the production was greatest from tissue cultures produced from mature embryos after 8 and 9 wk (Table 1). In these two treatments, an average of 3.2 transgenic tissue cultures were produced per bombardment, which was eight-fold higher than the production of 0.4 transgenic tissue cultures per bombardment from the 4-wk-old embryo explants. Additionally, the production frequency per bombardment from 8- and 9-wk-old tissue cultures was similar to the production of transgenic tissue cultures from microprojectile bombardment of immature embryo-derived callus (Torbert et al., 1995) indicating that the use of older tissue cultures initiated from mature embryos was unlikely to increase the yield of transgenic tissue cultures per microprojectile bombardment treatment. Based on these results further studies were conducted on 8- to 9-wk-old tissue cultures initiated from mature embryos.

Regeneration and Fertility of Transgenic Plants

To determine the regeneration capacity and fertility of plants produced from transgenic GAF/Park-1 mature embryo-derived tissue cultures, three microprojectile bombardment experiments were evaluated. Two experiments were the 8- and 9-wk tissue culture initiation treatments bombarded with pNGI reported in Table 1. The third experiment used pH24 bombarded into 8-wk-old callus. From a total of 46 microprojectile bombardment treatments that produced 85 transgenic tissue cultures, 49 regenerable tissue cultures were produced with an overall regeneration frequency of 58% and a range

Table 1. Effect of tissue culture age (weeks after explant isolation before microprojectile bombardment) on production of transgenic tissue cultures.

Mature embryos			Transgenic tissue cultures produced†		
Age (wk)	No. bombarded‡	No. bombardments	No.	Per mature embryo bombarded	Per bombardment
1	118	12	0	0	0
4	120	12	5	0.04	0.42
8	27	9	36	1.33	4.00
9	29	12	32	1.10	2.67

† Tissue cultures selected on paromomycin-containing medium were verified as transgenic based on detection of *npt II* gene product using ELISA.
‡ Approximately 0.6 g tissue was used per bombardment.

of 36 to 82% for the three experiments (Table 2). All plants regenerated from the 49 tissues cultures expressed the *npt II* gene product in leaf tissue as determined by NPT II ELISA indicating that they were transgenic. Positive ELISA tests of transgenic plants were distinctly yellow compared to tests conducted on wild-type non-transgenic plants and therefore could be scored visually. ELISA assays conducted on 10 T_0 plants regenerated from five different transgenic tissue cultures exhibited OD_{405} values corresponding to a range of 1.0 to 13.8 ng NPT II protein per gram fresh weight. The lowest ELISA OD_{405} value among them was more than 30 fold the average OD_{405} reading from assays of five non-transgenic plants.

Thirty-five tissue cultures regenerated at least one plant that produced seed resulting in a frequency of regenerable tissue cultures producing fertile, transgenic plants of 71% with a range of 43 to 100%. Thus, the overall production frequency was 0.76 tissue culture producing fertile, transgenic plants per microprojectile bombardment (Table 2) or 41% of transgenic tissue cultures producing fertile plants. Of the 29 fertile families produced by microprojectile bombardment with pNGI, 21 families produced progeny that segregated for the presence and absence of GUS activity in the endosperm (data not shown), six families produced only a few seed and were not assayed for GUS, and two families were GUS negative.

DISCUSSION

In oat, several tissue explants have been used for initiation of regenerable tissue cultures (Rines et al., 1992). Furthermore, several studies have focused on the mature embryo or tissues derived from it shortly after germination as a source of totipotent oat cells. However, none of these explants have been shown to produce transformable tissue cultures. In this study, friable, embryogenic tissue cultures were initiated from mature embryos at a frequency that was about half of the friable embryogenic callus initiation frequency previously obtained from immature embryos. Beyond the requirement of isolating more mature embryos to produce similar amounts of tissue culture, the procedures for callus initiation from mature and immature embryos of oat are essentially the same. The major advantage of the mature embryo for initiation of transformable tissue cultures is its convenience as an immediately available explant which can be inexpensively produced in large quantities. The convenience in isolating the mature embryo offsets the lower embryogenic tissue culture initiation frequency compared with immature embryos. A further advantage of mature embryos as a tissue culture explant is related to the physiological uniformity of mature seed versus immature embryos. Initiation of tissue cultures from all plants species is dependent on combinations of variables including source, physiological state and genotype of the explant, culture medium composition, and culture conditions. In this study, the mature embryo explant was isolated from the caryopsis after 16 h imbibition presumably reducing the capacity for variability in physiological state that could contribute to the tissue culture initiation frequency from this explant. In contrast, initiation of immature embryo-derived tissue cultures is subject to large variations in physiological condition of the donor plants. Moreover, donor plants are expensive to grow, and immature embryo isolation is laborious. Seed development along a panicle in oat tends to be less synchronous than seed development in spikes of wheat and barley or ears of maize. This increases the labor and expense of initiating tissue cultures from immature embryos of oat because only a few immature embryos can be isolated from an individual panicle. It seems likely that the identification of the mature embryo as a convenient explant for initiation of transformable tissue cultures will stimulate investigation of different oat genotypes for culture initiation and different media and culture conditions for improved tissue culture initiation.

The production of fertile, transgenic plants via microprojectile bombardment of tissue cultures derived from mature embryos in this study, at frequencies similar to those produced from immature embryo-derived callus, should facilitate further improvements in the oat transformation system. Tissue cultures initiated from mature embryos bombarded 8 to 9 wk after mature embryo

Table 2. Tissue culture lines producing fertile, transgenic plants following microprojectile bombardment of mature embryo-derived tissue cultures.

	No. transgenic tissue cultures†			Tissue cultures producing fertile transgenic plants per bombardment
Exp.	Total	Plant regeneration	Fertile plants	
1	36	23	17	17/9 = 1.89
2	32	12	12	12/12 = 1.00
3	17	14	6	6/25 = 0.24
Overall	85	49 (58)‡	35 (71)	35/46 = 0.76

† Tissue cultures and plants were verified as transgenic based on detection of the *npt II* gene product in callus and T_0 leaf tissue.
‡ Numbers in parentheses indicate percentages.

plating produced the highest frequency of transgenic tissue cultures (Table 1). This represents a 3- to 4-wk decrease in total duration of the tissue culture period before bombardment compared with the shortest total tissue culture period before bombardment used in our previous transformation system based on immature embryos (Somers et al., 1992; Torbert et al., 1995). A compilation of oat transformation experiments conducted using immature embryo-derived callus indicates that 34% of the 319 transgenic tissue cultures produced fertile plants (Torbert and Somers, 1995, unpublished results), whereas 41% of the mature embryo-derived transgenic tissue cultures isolated produced fertile, transgenic plants. Thus, use of the mature embryo-derived tissue cultures was at least similar in production of fertile, transgenic plants compared to the immature embryo system. Accordingly, we recommend a system for oat transformation in which mature embryos are isolated on a routine basis throughout the year to maintain a constant supply of transformable 8- to 9-wk-old tissue cultures for microprojectile bombardment. We plate about 100 mature embryos per experiment. Of the tissue cultures initiated after 8 to 9 wk, approximately 30 tissue cultures per experiment have the appearance of those shown in Fig. 1A and are used for microprojectile bombardment.

An interesting question regarding the use of mature embryos for transformation is whether tissue culture age can be further reduced before DNA delivery treatments, thereby further increasing regeneration capacity and fertility of regenerated plants. In other cereals, the scutellum of immature and mature embryos can be bombarded directly to produce transgenic tissue cultures, thus significantly decreasing the tissue culture period before bombardment. In oat, where the scutellum of immature embryos is not responsive, other sources of totipotent cells are required. In this study, five transgenic tissue cultures were produced from bombardment of mature embryos 4 wk after plating, but only one regenerated and produced fertile plants. This result is somewhat expected because we bombarded all explants whether they would initiate tissue culture or not, and we did not select for tissue culture appearance, and therefore regeneration capacity, before bombardment. Furthermore, the yield of transgenic tissue cultures from this treatment was much less than from bombardment of tissue cultures isolated 8 and 9 wk after mature embryo plating. Nevertheless, further effort is warranted in attempting to reduce the duration of tissue culture before DNA delivery. The convenience of mature embryo-derived callus will likely stimulate investigation along these lines.

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REFERENCES

Bregitzer, P.P., S.K.C. Milach, H.W. Rines, and D.A. Somers. 1995. Somatic embryogenesis in oat (*Avena sativa* L.) p. 53–62. *In* Y.P.S. Bajaj (ed.) Biotechnology in agriculture and forestry, Vol. 31. Somatic embryogenesis and synthetic seed. Springer-Verlag, Berlin.

Bregitzer, P., D.A. Somers, and H.W. Rines. 1989. Development and characterization of friable, embryogenic oat callus. Crop Sci. 29:798–803.

Chen, H., G. Xu, D.C. Loschke, L. Tomaska, and B.G. Rolfe. 1995a. Efficient callus formation and plant regeneration from leaves of oats (*Avena sativa* L.). Plant Cell Rep. 14:393–397.

Chen, Z., Q. Zhuge, and C. Sundqvist. 1995b. Oat leaf base: tissue with an efficient regeneration capacity. Plant Cell Rep. 14:354–358.

Christou, P., T.L. Ford, and M. Kofron. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into mature zygotic embryos. Bio/Technology. 9:957–962.

Cooley, J., T. Ford, and P. Christou. 1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor. Appl. Genet. 90:97–104.

Cummings, D.P., C.E. Green, and D.D. Stuthman. 1976. Callus induction and plant regeneration in oats. Crop Sci. 16:465–470.

Gana, J.A., G.C. Sharma, A. Zipf, S. Saha, J. Roberts and D.M. Wesenberg. 1995. Genotype effects on plant regeneration in callus and suspension cultures of *Avena*. Plant Cell, Tissue Org. Cult. 40:217–224.

Gless, C., H. Lorz, and A. Jahne-Gartner. 1996. Development of a highly efficient in vitro regeneration system from leaf bases of oat and barley as a prerequisite for transformation experiments. p. 410–411. *In* A. Slinkard et al. (ed.) Proceedings of the V International Oat Conference and the VII International Barley Genetics Symposium, Poster Session Vol 2. University Extension Press, Univ. of Saskatchewan, Saskatoon.

Heyser, J.W., and M.W. Nabors. 1982. Long term plant regeneration, somatic embryogenesis, and green spot formation in secondary oat (*Avena sativa*) callus. Z. Pflanzenphysiol. 107:153–160.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387–405.

King, I.P., H. Thomas, and P.J. Dale. 1986. Callus induction and plant regeneration from oat cultivars. p. 46–47. *In* D.A. Lawes and H. Thomas (ed.) Proc 2nd Int. Oats Conf., Aberystwyth, Wales. 15–18 July 1985. Martinus Nijhoff Publ., Dordrecht, Netherlands.

Klein, T.M., L. Kornstein, J.C. Sanford, and M.E. Fromm. 1989. Genetic transformation of maize cells by particle bomardment. Plant Physiol. 91:440–444.

Kosugi, S., Y. Ohashi, K. Nakajima, and Y. Arai. 1990. An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppressed endogenous β-glucuronidase activity. Plant Sci. 70:133–140.

Lorz, H., C.T. Harms, and I. Potrykus. 1976. Regeneration of plants from callus in *Avena sativa* L. Z. Pflanzenzuchtg. 77:257–259.

McCoy, T.J., R.L. Phillips, and H.W. Rines. 1982. Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue culture: High frequency of partial chromosome loss. Can. J. Genet. Cytol. 24: 37–50.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473–497.

Nabors, M.W., J.W. Heyser, T.A. Dykes, and K.J. DeMott. 1983. Long duration, high-frequency plant regeneration from cereal tissue culture cells. Planta 157:385–391.

Nabors, M.W., C.S. Kroskey, and D.W. McHugh. 1982. Green spots are predictors of high callus growth rates and shoot formation in normal and salt stressed tissue cultures of oat (*Avena sativa* L.). Z. Pflanzenphysiol. 105:341–349.

Nehra, N.S., R.N. Chibbar, N. Leung, K. Caswell, C. Mallard, L. Steinhauer, M. Baga, and K.K. Kartha. 1994. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5:285–297.

Rines, H.W., S.S. Johnson, and R.L. Phillips. 1986. Tissue culture induced variation in oats. p. 34–38. *In* D.A. Lawes and H. Thomas (ed.) Proc 2nd Int. Oats Conf, Aberystwyth, Wales. 15–18 July 1985. Martinus Nijhoff Publ., Dordrecht, Netherlands.

Rines, H.W. and H.H. Luke. 1985. Selection and regeneration of toxin insensitive plants from tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae*. Theor. Appl. Genet. 71:16–21.

Rines, H.W., and T.J. McCoy. 1981. Tissue culture initiation and plant regeneration in hexaploid species of oats. Crop Sci. 21:837–842.

Rines, H.W., R.L. Phillips, and D.A. Somers. 1992. Applications of tissue culture to oat improvement. p. 777–791. *In* H.G. Marshall and M.E. Sorrells (ed.) Oat Science and technology. Agron. Monogr. 33. ASA, Madison, WI.

Sivamani, E., P. Shen, N. Opalka, R.N. Beachy, and C.M. Fauquet. 1996. Selection of large quantities of embryogenic callus from indica rice seeds for production of fertile transgenic plants using the biolistic method. Plant Cell Rep. 15:322–327.

Somers, D.A., H.W. Rines, W. Gu, H.F. Kaeppler, and W.R. Bushnell. 1992. Fertile, transgenic oat plants. Bio/Technology 10:1589–1594.

Somers, D.A., H.W. Rines, K.A. Torbert, W.P. Pawlowski, and S.K.C. Milach. 1996. Genetic transformation in *Avena sativa* L. (oat). p. 178–190. *In* Y.P.S. Bajaj (ed.) Biotechnology in agriculture and forestry, Vol. 38, Plant protoplasts and genetic engineering VII. Springer-Verlag Berlin, Heidelberg.

Torbert, K.A., H.W. Rines, and D.A. Somers. 1995. Use of paromomycin as a selective agent for oat transformation. Plant Cell Rep. 14:635–640.

Vain, P., M.D. McMullen, and J.J. Finer. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12:84–88.

Vasil, V., V. Srivastava, A.M. Castillo, M.E. Fromm, and I.K. Vasil. 1993. Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology 11: 1553–1558.

Wan, Y., and P.G. Lemaux. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104:37–48.

Weeks, J.T., O.D. Anderson, and A.E. Blechl. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol. 102:1077–1084.

Chromosomal Location of Genes for Resistance to Karnal Bunt in Wheat

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ABSTRACT

Karnal bunt (*Tilletia indica* Mitra) infestation of wheat (*Triticum aestivum* L.) kernels reduces grain quality. Deployment of genetic resistance would be preferable to chemical applications for control of the disease. Inoculation studies were carried out in a wheat mapping population with the aim of locating genes for resistance. Recombinant inbred (RI) lines from a cross between a resistant synthetic wheat (*Triticum turgidum* 'Altar 84' × *T. tauschii*) and the susceptible common wheat cultivar 'Opata 85' were inoculated with Karnal bunt sporidial suspension and evaluated for symptom development in the field for three seasons and in the greenhouse. Based on restriction fragment length polymorphism (RFLP) analyses, regions on chromosome arms 3BS and 5AL carrying marker alleles from the Altar durum parent were consistently associated with reduced kernel disease. Main marker effects accounted for up to 32% of disease variation in the field but only 15% in the greenhouse, where the level of disease was higher, suggesting an environmental component of resistance. The tagging of these Karnal bunt partial-resistance genes in tetraploid and hexaploid backgrounds may facilitate the accumulation of resistance via marker-assisted transfer to susceptible durum and common wheat cultivars. This practice should reduce laborious disease screening requirements.

Karnal or partial bunt (KB) was first identified in 1931 in wheat fields near Karnal, India. Susceptible wheat cultivars are attacked via floral infection by seed-, air-, or soil-borne sporidia, resulting in partial replacement of kernels with masses of teliospores. These impart a foul odor to the grain and reduce its fitness for consumption. The principal wheat-growing areas affected by KB include northwestern India, Pakistan, and northwestern Mexico. The disease appeared in the Yaqui Valley of Mexico in the early 1970s and recently has caused problems in the U.S. states of Arizona and California (Anonymous, 1996).

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Chemical control of the disease with fungicides applied near flowering stage is only partially effective owing to the varied modes of spore transmission. It is commercially impractical (Fuentes-Dávila and Rajaram, 1994) because of the zero tolerance level for KB spores imposed by importing countries. Development of resistant cultivars is the preferred control measure. The main sources of resistance in CIMMYT's breeding program have been Chinese, Indian, and Brazilian wheats (Fuentes-Dávila and Rajaram, 1994).

Durum wheat (*Triticum durum* Desf.) cultivars typically show higher levels of field resistance to KB than bread wheat (Gill et al., 1981; Gill et al., 1983). Under spray inoculation, which mimics natural attack, both durums and triticales (× *Triticosecale* Wittmack) showed less disease than bread wheats (Warham, 1988). However, the more severe boot inoculation technique (described below) gave similar disease levels across crops, suggesting that field resistance is partly morphological. Bread wheat (genome formula AABBDD) is an ancestral hybrid between tetraploid durum wheat (*T. turgidum*, genome formula AABB) and the wild diploid *Aegilops squarrosa* L. (= *Ae.* or *T. tauschii*, genome formula DD). In crosses between durums and bread wheats followed by successive backcrosses to wheat, higher KB susceptibility accompanied restoration of the wheat spike morphology (Gill et al., 1983).

Homoeology of the wheat genomes with those of wild and cultivated diploids in the tribe Triticeae makes the latter, especially *Ae. squarrosa*, ready sources of genes for wheat improvement. Accessions of several *Aegilops* species were screened for KB resistance under greenhouse conditions (Warham et al., 1986) via boot inoculation. In nine species, all tested accessions showed complete resistance. It was also observed in five of 12 *Ae. squarrosa* accessions. Among cultivated diploid Triticeae, all barley (*Hordeum vulgare* L.) and many rye (*Secale cereale* L.) accessions proved highly KB-resistant (Warham, 1988), suggesting their potential use as resistance sources.

Abbreviations: RFLP, restriction fragment length polymorphism; RI, recombinant inbred; KB, Karnal bunt.

Shoot meristem: an ideal explant for *Zea mays* L. transformation

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Abstract: We report on a rapid high-frequency somatic embryogenesis and plant regeneration protocol for *Zea mays*. Maize plants were regenerated from complete shoot meristem (3–4 mm) explants via organogenesis and somatic embryogenesis. In organogenesis, the shoot meristems were directly cultured on a high-cytokinin medium comprising 5–10 mg·L^{-1} 6-benzylaminopurine (BAP). The number of multiple shoots produced per meristem varied from six to eight. Plantlet regeneration through organogenesis resulted in just four weeks. Callus was induced in five days of incubation on an auxin-modified Murashige and Skoog (MS) medium. Prolific callus, with numerous somatic embryos, developed within 3–4 weeks when cultured on an auxin medium containing 5 mg 2,4-dichlorophenoxyacetic acid·L^{-1}. The number of multiple shoots varied from three to six per callus. Using R23 (Pioneer, Hi-Bred, Johnston, Iowa), the frequency of callus induction was consistently in excess of 80% and plant regeneration ranged between 47 and 64%. All regenerated plantlets survived in the greenhouse and produced normal plants. Each transgenic plant produced leaves, glumes, and anthers that uniformly expressed green fluorescent protein (GFP). The *GFP* gene segregated in the pollen. Based on this data it is concluded that the transgenics arose from single-cell somatic embryos. The rate of transfer DNA (T-DNA) transfer to complete shoot meristems of *Zea mays* was high on the auxin medium and was independent of using super-virulent strains of *Agrobacterium*.

Key words: Zea mays, shoot meristems, organogenesis, embryogenesis, *Agrobacterium*-mediated transformation.

Résumé : Les auteurs rapportent un protocole rapide et efficace d'embryogenèse somatique et de régénération chez le maïs, *Zea mays*. Des plants de maïs ont été régénérés à partir d'explants de méristème apical (3–4 mm) par organogenèse ou par embryogenèse somatique. En organogenèse, les méristèmes apicaux ont été directement mis en culture en présence d'un milieu à forte concentration en cytokinines (5–10 mg de 6-benzylaminopurine·L^{-1}). Le nombre de tiges multiples produites par méristème variait entre six et huit. La régénération de plantules par organogenèse était réalisée en seulement quatre semaines. Des cals étaient induits en incubant cinq jours sur un milieu MS modifié contenant des auxines. Des cals prolifiques avec de nombreux embryons somatiques se sont développés en 3–4 semaines lorsque placés sur un milieu avec auxines (contenant 5 mg d'acide 2,4-dichlorophénoxyacétique·L^{-1}). Le nombre de tiges multiples variait entre trois et six par cal. Avec le génotype R23 (Pioneer), la fréquence d'induction de cals excédait régulièrement 80 % et la régénération variait entre 47 et 64 %. Toutes les plantules régénérées ont survécu en serre et ont produit des plantes normales. Chaque plante transgénique a produit des feuilles, des glumes et des anthères exprimant de manière uniforme la protéine fluorescente verte (GFP). Le gène codant pour la GFP était en ségrégation au sein du pollen. Sur la base de ces données, les auteurs concluent que les plantes transgéniques ont été produites à partir d'embryons somatiques unicellulaires. Le taux de transfert de l'ADN-T chez des méristèmes apicaux complets du *Zea mays* était élevé sur le milieu contenant de l'auxine et cela indépendamment de l'emploi de souches hypervirulentes de l'*Agrobacterium*.

Mots clés : Zea mays, méristèmes apicaux, organogenèse, embryogenèse, transformation agrobactérienne.

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Introduction

Corn can be regenerated in tissue culture and transformed using a variety of tissues. Explants used in previous studies include immature embryos, immature inflorescences, protoplasts, anthers, microspores, shoot apices, and suspension cultures. Regeneration from corn cultures was achieved through organogenesis and somatic embryogenesis (Harms et al. 1976; Potrykus et al. 1977; Rhodes et al. 1988; Vasil et al. 1984; Vasil and Vasil 1986; Cai et al. 1988; Prioli and Sondhal 1989; Tomes and Smith 1985; Lu et al. 1982; Novak et al. 1983; Armstrong and Green 1985). However, concomitant with the use of these regeneration protocols are severe limitations.

Common problems associated with regeneration of maize from immature embryos, immature inflorescences, shoot apices, and embryogenic suspension culture are restrictions associated with genotype specificity, somaclonal variation, chimeras, difficulties in maintaining totipotency for extended periods of time, and low frequencies of callus induction and plant regeneration (Vasil et al. 1985; Vasil and Vasil 1987; Lu et al. 1983; Tomes and Smith 1985). As a consequence, the suitability of these explants and cell cultures for efficient production of transgenics is likewise limited. These restrictions with respect to transgenic plant production also apply to commercially important dicotyledons like soybeans and to cereals such as rice, wheat, barley, and corn.

Here we report that *Agrobacterium tumefaciens* efficiently transfers transfer DNA (T-DNA) to complete *Zea mays* shoot meristems. Based on these observations, we can say that T-DNA transfer to shoot meristems is high, ranging between 60 and 87%, and that it is not dependent on the use of supervirulent *Agrobacterium* strains (Hiei et al. 1994; Ishida et al. 1996). This frequency of transfer to the shoot meristem is magnitudes higher than previously reported when using isolated maize shoot apices (Gould et al. 1991) and the number of cells per explant expressing screenable marker genes such as those coding for β-D-glucuronidase (*GUS*) or green florescence protein (*GFP*) is also much higher than suggested in previous reports. This study shows that by using complete shoot meristems *Zea mays* can be regenerated at a high frequency through either organogenesis or somatic embryogenesis and can also be efficiently transformed at high frequency using *Agrobacterium* strains.

Materials and methods

Maize genotypes

Six different hybrids and an inbred of corn were used for transformation. The hybrids LH 74 × A 641, LH 262 × LH 252, LH 198 × LH 227, FR 1064 × FR 1064 (SDMS) × LH 185, and LH 176 × LH 177 DMS were obtained from the Indiana Crop Improvement Association (Lafayette, Ind.) and the inbred line R23 ('Champaign White Pearl') was obtained from Pioneer Hi-Bred (Johnston, Iowa).

Agrobacterium strains and plasmids

Three different *Agrobacterium* strains, EHA105, LBA4404, and GV3101, were used. EHA105 is a supervirulent *Agrobacterium* that contains extra copies of the virulence genes *virB*, *vir*C, and *virG*. Plasmid pBI121 was transformed into EHA105 and LBA4404 (Chen and Vierling 2000). Covalently linked to the T-DNA is a selectable kanamycin gene, as well as a screenable *GUS* marker (Hood et al. 1986). Plasmid pMP90RK, containing the *GFP* gene that is driven by the rice actin promoter, was introduced into GV3101 (Koncz and Schell 1986).

Plant transformation method

Three different strains of *Agrobacterium tumefaciens* were grown overnight on LB medium supplemented with kanamycin and gentamycin (50 mg·L^{-1}each) with shaking (200 × *g*) at 26–28°C. The bacterial optical density (OD) was read on a Beckman spectrophotometer(Beckman Coulter, Fullerton, Calif.) at 660 nm. One hour before the *A. tumefaciens* reached the desired OD of 1–1.5, 200 µM acetosyringone was added. Following this, the cells were centrifuged at 2000 × *g* for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in resuspension medium (half-strength MS salts (Murashige and Skoog 1962) + 1% w/v glucose + 200 µM acetosyringone (pH 5.2)). Cell density was readjusted to an OD of 0.8 at 660 nm by diluting with resuspension medium.

The maize shoot meristems were incubated in the *A. tumefaciens* cell suspension for 3 h, plated on cocultivation medium (half-strength MS salts + 2% w/v glucose), and incubated in the dark for 3 to 4 days. Meristems were transferred to auxin-supplemented callus-induction media (Fig. 1*a*) containing carbenicillin (500 mg·L^{-1}) and cefotaxime (250 mg·L^{-1}). The cultures were reincubated in the dark and regularly subcultured every 15 days. Alternatively, the infected shoot meristems were grown on a modified MS medium containing 6-benzylaminopurine (BAP; 5–10 mg·L^{-1}) and kinetin (Kn; 0.5–2.0 mg·L^{-1}) and regenerated using organogenesis in the light.

In vitro regeneration methods

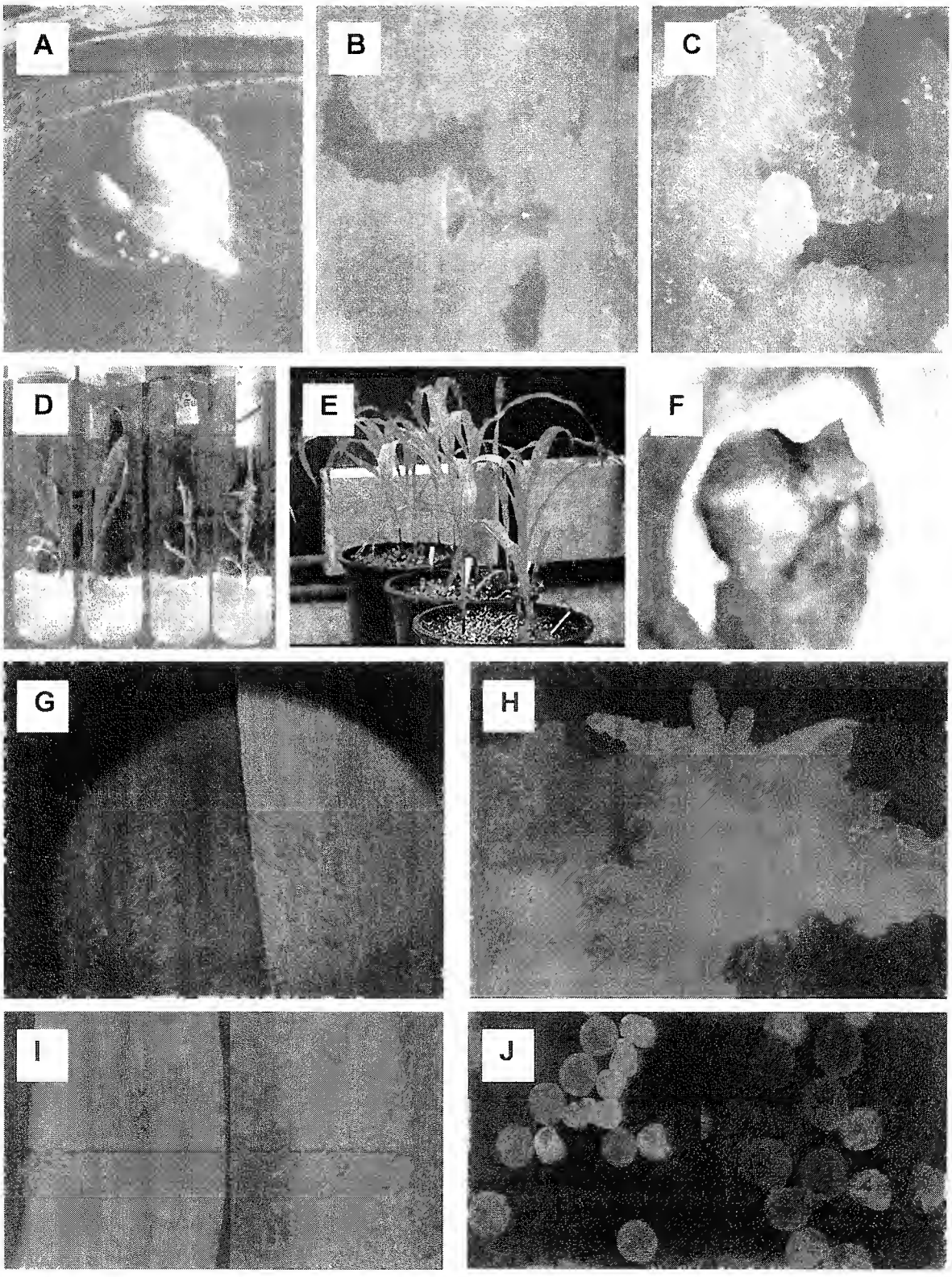
Regeneration via somatic embryogenesis

Prolonged incubation of meristem-derived callus on modified MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in proliferation of somatic embryos. Calli containing numerous somatic embryos were transferred to MS regeneration medium supplemented with myoinositol and glycine (100 mg·L^{-1}), in addition to Kn (1 mg·L^{-1}) and BAP (10 mg·L^{-1}), to form green plantlets that were then transferred to soil and acclimatized in standard greenhouse conditions.

Regeneration via organogenesis

Shoot meristems isolated from *Zea mays* seeds that were germinated on the modified MS medium containing auxin were cocultivated with *A. tumefaciens* for 3 days in the absence of light. After 3 days, the meristem cultures were transferred to a modified MS medium containing two cytokinins (0.5 mg Kn · L^{-1} + 5–10 mg BAP·L^{-1}) in addition to carbenicillin and cefotaxime (50–500 mg·L^{-1}) and incubated in the light.

Fig. 1. Transformation of R23 genotype with pBI121 and pMP90RK. (A) Three- to four-day-old germinating seedling. (B) Callus induction from shoot meristem. (C) Globular heart-shaped embryos. (D) Corn plantlets regenerated in vitro. (E) Transgenic plants growing in the greenhouse. (F) Transformed meristem expressing *GUS*. (G) Leaf tissue from greenhouse grown plants expressing *GFP* (right) and control (left). (H) Embryogenic callus expressing *GFP*. (I) Anther expressing *GFP* (left) and control (right). (J) Pollen expressing *GFP* (left) and control (right).



Analyses of gene expression

GUS gene histochemical and PCR analysis

The GUS assay developed by Jefferson (1987) was followed to assess gene expression in the primary transformants at 7-day intervals after infection and subsequently at 15-day intervals until the plants were transferred to the greenhouse. Leaves from 10 to 20 infected regenerants were tested for GUS activity along with uninfected controls. DNA was isolated from calli that were verified as being free of *A. tumefaciens* and from the regenerated plants using the procedure described by Dellaporta et al. (1983). PCR primers gusF (5′-ATGTTACGTCCTGTAGAAACCCCAAC-3′) and gusR (5′-TCATTGTTTGCCTCCCTGCTGCGG -3′) for the *gus*A gene were synthesized by Integrated DNA Technologies (Coralville, Iowa).

Analysis of GFP expression

GFP expression was monitored using an Olympus, SZX12 epifluorescence stereomicroscope equipped with an Olympus filter cube containing a 460–490 nm excitation filter and an emission filter 510 interference.

Southern blot analysis

Genomic DNA (10 μg) from regenerated plants was digested with *Bam*HI and *Sac*I to release the entire *gus*A fragment. The fragments were separated on 1% w/v agarose gels and transferred to positively charged nylon membranes (Roche, Basel, Switzerland). A PCR-generated, 1.2-kb *gus*A fragment was used as a probe and labeled with [α^{32}P]dCTP (110 TBq/mol) using a random primer DNA-labeling kit (Amersham Bioscience, Piscataway, N.J.) according to the manufacturer's instructions. A 1-kb DNA ladder (Invitrogen, Carlsbad, Calif.) was used as the DNA marker. Southern hybridization was carried out as per the standard procedure (Sambrook and Russel 2001). Membranes were placed on X-ray film (Kodak XAR-5) with intensifying screens at –70°C for 3 days to visualize the hybridization results.

Results

In vitro regeneration studies with *Zea mays*

Regeneration via organogenesis

Shoot meristems were cultured in light for induction of shoots via organogenesis. The 3- to 4-day-old germinating seedlings with complete shoot meristems were cultured on a modified MS medium supplemented with Kn (0.5 mg·L^{-1}) and BAP (5–10 mg·L^{-1}). Green shoot primordia developed from this explant in 10 days. In 4–6 weeks, complete shoots developed from the green primordia. The number of shoots generated by each explant varied from 6 to 8 and the frequency of shoot regeneration was 80–90%.

Regeneration via somatic embryogenesis

Shoot meristems from six different genotypes of maize were cultured in the dark for the induction of calli on modified MS medium supplemented with 100 mg myo-inositol·L^{-1} and 400 mg thiamine–HCl · L^{-1} in addition to 5 mg 2,4-D·L^{-1} (Fig. 1*a*). Callus initiation was observed on day 5 after transfer to the medium (Fig. 1*b*). The mean callus induction frequency in three independent experiments ranged from 86–94% (Table 1). The number of shoots per callus was high and varied from 3 to 6. Calli with numerous somatic embryos were formed within 10–15 days of incubation on auxin-supplemented medium (Fig. 1*c*). Calli containing somatic embryos were transferred to regeneration medium (MS medium supplemented with myo-inositol (100 mg·L^{-1}), glycine (100 mg·L^{-1}), Kn (1 mg·L^{-1}), and BAP (10 mg·L^{-1})) for maturation of somatic embryos. Green plantlets developed from the somatic embryos in 30 to 40 days following transfer to the germination medium (Fig. 1*d*). The plantlets were transferred to soil and hardened in the greenhouse (Fig. 1*e*). The plant regeneration frequency in all six genotypes tested was reproducible and ranged from 47 to 64%, thus indicating that the regeneration protocol is genotype independent. No albino or sectored plants were observed.

Agrobacterium-mediated transformation of complete shoot meristems

The expression of *GUS* was monitored following *Agrobacterium*-mediated transformation of shoot meristems, in meristem-derived calli, and in leaves of the regenerated plants. Figure 1*f* shows typical maize shoot meristem that formed calli and was stained for GUS activity 10 days post transformation. The leaves from the first 24 primary transformants were also tested for GUS activity. All of the leaves tested on each of the plants were positive for *GUS* expression.

In Table 2, the percentage of transformed shoot meristems is reported on a strain-by-strain basis. LBA 4404 contains the *GUS* gene. Following infection, it was successfully transferred to 86% of the shoot meristems. GV3101 carries the *GFP* gene. Here, T-DNA was transferred to 66% of the infected shoot meristems. Notably, the super-virulent strain EHA105 transferred *GUS* to only 60% of infected shoot meristems. Thus, efficient T-DNA delivery is independent of the use of supervirulent strains using this protocol. The control constructs that were *vir* competent but lacked either the *GUS* or *GFP* genes did not produce positive expression signals after *Agrobacterium*-mediated transformation. As expected, these shoots neither showed GFP fluorescence nor did they stain positive for GUS. GFP expression was constantly monitored from the 3rd day post transformation until seed production. GFP expression was uniform in all somatic tissues including the somatic embryos, regenerated plants, successive leaves, glumes, and anthers (Figs. 1*g*–1*j*). The pollen segregated 1:1 with respect to the expression of GFP fluorescence.

Twenty-day-old corn calli, as well as regenerated plants, were randomly chosen for Southern blot analysis. The predominant unit length hybridization signal concentrated as predicted at 1.8 kb when corn genomic DNA was double digested with *Bam*HI and *Sac*I. DNA isolated from non-transformed plants did not hybridize with the *GUS* probe. The data from Southern hybridization has confirmed the transformation data compiled based on PCR and histochemical assays for GUS. Figure 2 shows the PCR and Southern analysis of *GUS* in 12 transgenic plants.

Discussion

Several prerequisites exist for developing an efficient

Table 1. Means for callus-induction and plant-regeneration frequencies using shoot meristems in *Zea mays*.

Hybrid	Genotype	No. of shoot meristems	No. of calli	No. of shoots	Callus-induction frequency	Shoots/callus	Regeneration (%)
1	R 23	161.67	151.67	72.33	93.74	5.67	48.73
2	LH74 XA641	100	88.33	41.67	88.64	4.33	47.39
3	LH 262 × LH252	61.67	55.67	28.67	90.00	4.67	52.44
4	LH 198 × LH 227	70	60.00	38.33	86.11	3.67	64.16
5	LH 176 × LH 177 DMS	81.67	71.67	42.00	88.01	3.67	58.80
6	FR 1064 × FR 1064 (SDMS) × LH 185	50	44.00	24.33	88.00	4.33	55.29
Mean		87.50	78.56	41.22	89.08	4.39	54.47
±SE		7.84***	6.96***	3.78***	2.48 n.s.	0.68 n.s.	6.50 n.s.
CV (%)		15.50	15.30	15.90	4.80	26.90	20.7

Note: ***, significant at $P \leq 0.001$; n.s., not significant; SE, standard error.

Table 2. Summary of transformation experiments with different *Agrobacterium* strains using complete shoot meristems of *Zea mays*.

Experiment	*Agrobacterium*	No. of shoot meristems	No. of plants tested for *GUS*	No. of plants expressing *GUS/GFP* activity and positive for PCR	*GUS/GFP* frequency (%)
1	LBA 4404	80	15	13	86.70
2	GV 3101	75	12	8	66.70
3	EHA 105	75	15	9	60.00

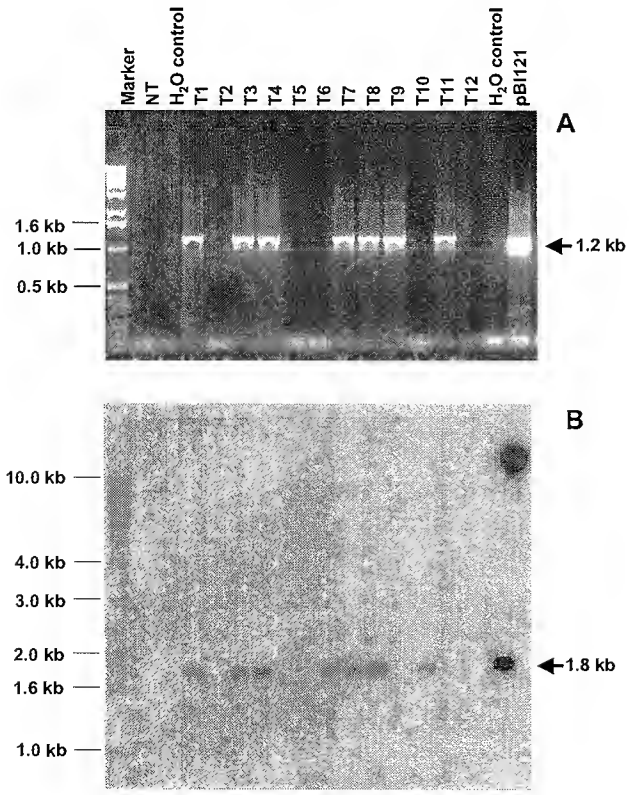


Fig. 2. Analysis of R23–pBI121 transgenic plants by PCR and Southern blotting. PCR amplification was targeted to a part of the *gus*A gene. For Southern hybridization, the genomic DNA from the same plants and pBI121 were digested with *Sac*I and *BamH*I and hybridized to a probe specific for the *gus*A gene. NT is DNA from non-transformed plants and lanes marked as H_2O control in panel A correspond to empty lanes in panel B in which no sample was loaded. Marker lanes in panel A and B represent 1-kb ladder DNA markers from Invitrogen.

transformation system in any explant. These include identification of a transformable explant, along with its subsequent rapid in vitro proliferation leading to dependable regeneration from competent transgenic cells. In this paper, we report the development of high-frequency callus induction, plant regeneration, and a high incidence of T-DNA transfer to complete maize shoot meristems.

The time required for callus induction using either immature embryos or inflorescences is reduced from 12 days to 6 when complete shoot meristems are cultured on the medium described here and incubated in the dark. Likewise, the timeline for the induction of prolific somatic embryogenesis is significantly reduced. When callus from complete shoot meristems is continuously cultured on a modified MS medium containing auxin, somatic embryos are formed within 28–30 days. In contrast, somatic embryos obtained from callus derived from immature embryos and inflorescences required 60–75 days with repeated sub-culturing and changes in hormone regime. In this scenario, auxin is removed and replaced with cytokinin. The use of complete shoot meristems as sources of callus and somatic embryos appears to be universal among the grasses (Sairam et al. 2002). Given the rapid formation of somatic embryos, the time needed to recover regenerated plants is reduced. Typically, plants derived from immature embryos require between 4 and 6 months to mature. In contrast, using the somatic embryos derived from meristem-based callus regeneration occurs in 4–6 weeks.

Based on these results, it appears that rapid, high-frequency callus and somatic embryogenesis may be a property of undifferentiated cells that in turn contain receptors particularly responsive to auxin. In this scenario, early embryo development has been shown to depend on the activity of the polar auxin transport system (Hobble 1998). Analyses of *fass* and *monoopteros* mutants of *Arabidopsis* clearly demonstrate the significance of auxin in early embryo development (Fisher et al. 1996).

The cells of shoot meristem not only serve as an excellent source of regeneration material but also contain competent cells that function efficiently with respect to T-DNA transfer. Depending on the *Agrobacterium* strain used, T-DNA was transferred at rates ranging fom 60 to 87% following infection of maize shoot meristems. Transfer rates were calculated based on the number of meristem explants expressing either *GUS* or *GFP* divided by the number of explants infected. High transfer rates are independent of the use of supervirulent strains. This is a significant observation, because nearly all previously reported transformation protocols required the use of supervirulent vectors and yield only a small percentage of transformants. Equally notable is the fact that *Agrobacterium* infection does not reduce the number of regenerable explants.

In this paper, we also report that transgenic plants may be produced rapidly via direct organogenesis. By eliminating the callus step, the time needed to produce a regenerated plant in tissue culture is significantly reduced. As a consequence, the incidence of both retro and DNA transposon mobilization are likewise abridged. This is no small consideration with respect to the design of a regeneration and (or) transformation system. The movement of transposons or copies thereof from one location on a chromosome to another often results in their insertion into other genes. Because the reading frame of that gene is interrupted, expression ceases and function is lost. Thus, transposon movement drastically changes both the genetic signature and the expression patterns of the transformed cells, and the resulting new transgenics differ by more than the single gene that was originally delivered to the competent cells.

Transposon mutagenesis is but one of the problems associated with plant regeneration. Specifically, plants regenerated following organogenesis do not always arise from single cells, a fact that has been reported by Gould et al. (1991), among others. In this case, a high incidence of chimeras was reported following *Agrobacterium*-mediated transformation of isolated shoot apices.

Plant regeneration and T-DNA delivery to different maize cultivars reported here suggest that this protocol is genotype independent. This conclusion is also in agreement with our previous work in *Tripsacum* (Sairam et al. 2002). The *Tripsacum* cultivar Pete transforms and regenerates at a high rate using complete shoot meristems as the starting material. 'Pete' was established as a composite strain that originated from natural stands of gamma grass in Kansas and Oklahoma (Kidinger and DeWald 1991). Seventy original seed lots were bulked and advanced through the third generation by open pollination and then combine harvested. Kidinger and Vierling (1994) analyzed 19 isozyme patterns and confirmed the presence of multiple alleles at multiple loci, thus confirming the genetic heterogeneity of this population.

In this paper, we report that the rate of T-DNA transfer to complete shoot meristems of *Zea mays* is high on the auxin medium and is independent of the use of supervirulent *Agrobacterium* strains. Moreover, these explants can be efficiently used to regenerate plants via somatic embryogenesis and direct organogenesis. These results are consistent with what has been reported earlier for the forage grass *Tripsacum* (Sairam et al. 2002). The fact that complete shoot meristems contain an increase in the number of competent cells following prolonged auxin incubation suggests that complete shoot meristems can be used as target cells for *Agrobacterium*-mediated transfer to the other related cereals.

Finally, based on the evidence presented here, it may be concluded that T_0 plants are heterozygous for the *GFP* gene and arose from single-cell somatic embryos. As shown in Fig. 1*d*, plantlets from transgenic callus uniformly and without exception expressed GFP. Moreover, each successive mature leaf uniformly expresses GFP. Following the transition of somatic to floral meristems, it is expected that the expression of GFP will be uniform in tissues such as the glumes and anthers. In contrast, GFP expression should segregate in the pollen of a transgenic plant (Fig. 1*j*). Indeed, a 1:1 segregation was observed in these plants and is consistent with the integration of a single *GFP* gene in a single cell following *Agrobacterium tumefaciens* mediated transformation. The transfer and expression data for *GUS* is consistent with the Southern positives observed in T_0 calli and T_0 plants. Collectively, these observations provide strong evidence that production of T_0 transgenic plants are frequent, stable, and of single-cell origin.

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References

Armstrong, C.L., and Green, C.E. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta, **164**: 207–214.

Cai, Q., Kuo, C., Qian, Y., Jiong, R., and Zhou, Y. 1988. Somatic embryogenesis and plant regeneration from protoplast of maize (*Zea mays* L.) *In* Progress in plant protoplast research. Proceedings of the 7th International Protoplast Symposium, 6–11 December, Wagenigen, The Netherlands. *Edited by* K.J. Puite, J.J.M. Dons, H.J. Huizing, A.J. Kool, M. Koornneef, and F.A. Krens. Klewer Academic Publishers, Dordrecht, The Netherlands.

Chen, H., and Vierling, R.A. 2000. Molecular cloning and characterization of soybean peroxidase gene families. Plant Sci. **150**: 129–137.

Dellaporta, S.L., Wood, L., and Hicks, J.B. 1983. A plant DNA mini preparation: version 11. Plant Mol. Biol. Rep. **1**: 19–21.

Fisher, R.H., Barton, M.K., and Cohen, T.J. 1996. Hormonal studies of *fass*, an *Arabidopsis* mutant that is altered in organ elongation. Plant Physiol. **110**: 1109–1121.

Gould, J., Devey, M., Hasegawa, O., Ulian, E.C., Peterson, G., and Smith, R.H. 1991. Transformation of *Zea Mays* L. using *Agrobacterium* tumefaciens and the shoot apex. Plant. Physiol. **95**: 426–434.

Harms, C.T., Lorz, H., and Potrykus, I. 1976. Regeneration of plantlets from callus cultures of *Zea mays*. Z. Pflanzenzuecht. **77**: 347–351.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. **6**: 271–282.

Hobble, L.J. 1998. Auxin: molecular genetic approaches in *Arabidopsis*. Plant Physiol. Biochem. **36**: 91–102.

Hood, E.E., Chilton, S.W., Chilton, M.D., and Fraley, R.T. 1986. T-DNA and opine synthetic loci in tumors incited by *Agrobacterium tumefaciens* A281 on Soybean and *Alfalfa* plants. J. Bacteriol. **168**: 1283–1290.

Ishida, Y., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat. Biotechnol. **14**: 745–750.

Jefferson, R.A. 1987. Assaying chimeric genes: the *GUS* gene fusion system. Plant Mol. Biol. Rep. **5**: 387–405.

Kidinger, B., and Vierling, R.A. 1994. A method to enhance germination of eastern gamagrass. Maydica, **39**: 1–4.

Kidinger, B., and Dewald, C.L. 1997. The reproductive versatility of eastern gamagrass. Crop Sci. **37**: 1351–1360.

Koncz, C., and Schell, J. 1986. The promoter of TL DNA gene controls the tissue specific expression of chiameric genes carried by a novel type of *Agrobacterium* vector. Mol. Gen. Genet. **204**: 383–396.

Lu, C., Vasil, I.K., and Ozian-Akins, P. 1982. Somatic embryogenesis in *Zea mays* L. Theor. Appl. Genet. **62**: 109–112.

Lu, C., Vasil, V., and Vasil, I.K. 1983. Improved efficiency of somatic embryogenesis in tissue culture of maize (*Zea mays* L.). Theor. Appl. Genet. **66**: 285–289.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. **15**: 473–497.

Novak, F.J., Dolezelova, M., Nesticky, M., and Piovarci, A. 1983. Somatic embryogenesis and plant regeneration in *Zea mays* L. Maydica, **28**: 381- 390.

Potrykus, I., Harms, C.T., Lorz, H., and Thomas, E. 1977. Callus formation from stem protoplasts of corn. Mol. Gen. Genet. **156**: 347–350.

Prioli, L.M., and Sondahl, M.R. 1989. Plant recognition and recovery of fertile plants from protoplasts of maize (*Zea mays* L.) Bio/Technology, **7**: 589–594.

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., and Detmer, J.J. 1988. Genetically transformed maize plants from protoplasts. Science (Washington, D.C.), **240**: 204–207.

Sairam, R.V., Wilber, C., Franklin, J., Smith, B., Brazil, J., Hassel, R., Whaling, D., Frutiger, K., Blakey, C.A., Vierling, R., and Goldman, S.L. 2002. High-frequency callus induction and plant regeneration in *Tripsacum dactyloides* (L.). In Vitro Cell. Dev. Biol. Plant, in press.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, N.Y.

Tomes, D.T., and Smith, O.S. 1985. The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. Theor. Appl. Genet. **70**: 505–509.

Vasil, V., and Vasil, I.K. 1986. Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L. J. Plant Physiol. **124**: 399–408.

Vasil, V., and Vasil, I.K. 1987. Formation of callus and somatic embryos from protoplasts of a commercial hybrid of maize (*Zea mays* L.). Theor. Appl. Genet. **73**: 793- 798.

Vasil, V., Vasil, I.K., and Lu, C. 1984. Somatic embryogenesis in long-term callus cultures of *Zea mays* L. (Gramineae). Am. J. Bot. **71**: 158–161.

Vasil, V., Vasil, I.K., and Lu, C. 1985. Histology of somatic embryogenesis in cultured immature embryos of maize (*Zea mays* L.). Protoplasma, **127**: 1–8.